# Comparative Ototoxicity of Gentamicin and Amikacin and the Otoprotective Effects of Total Glucoside of Paeony

Pratyush Kayastha

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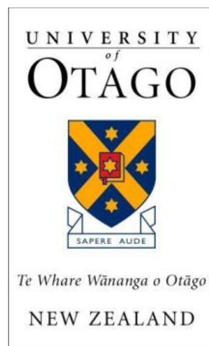

## Abstract

Despite their ototoxic potential, aminoglycosides are commonly used for the treatment of serious gram-negative bacterial infections such as cystic fibrosis and life-threatening conditions such as sepsis. Currently, there is no effective treatment for the adverse side effects that can develop, such as aminoglycoside-induced ototoxicity, resulting in permanent hearing loss and balance disorders. Moreover, *in-vitro* studies that have evaluated and compared aminoglycoside-induced toxicity in both the cochlea and vestibular system are limited. Therefore, to delineate aminoglycosides' toxic profile, the cochlear and vestibular toxicity of the aminoglycosides, gentamicin and amikacin, were evaluated and compared simultaneously using an *in-vitro* rat cochlear and utricular explant culture model. For this purpose, the inner ear explants were treated with different concentrations of aminoglycosides (0.3 - 2.4 mM) and the hair cell loss was evaluated using a fluorescent microscope. As inflammation is one of the relatively unexplored mechanisms behind aminoglycoside-induced toxicity, the otoprotective potential of an anti-inflammatory agent, Total Glucoside of Paeony (TGP) (100 µg/ml), was assessed following the aminoglycoside treatment. Gentamicin was found to be more toxic to both the cochlea and vestibular system than amikacin, and relatively greater gentamicin and amikacin-induced damage was observed in the cochlea compared to the vestibular system. Interestingly, the TGP treatment was found to ameliorate aminoglycoside-induced vestibular but not cochlear toxicity so, the differences between the underlying mechanisms of aminoglycoside-induced inflammation in the cochlear and vestibular systems are deemed worthy of further study.

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# List of abbreviations

| Abbreviation | Definition |
|---|---|
| ABR | Auditory nerve brainstem evoked responses |
| BSA | Bovine serum albumin |
| CNS | Central Nervous System |
| CX3CL1 | Fractalkine |
| DMEM | Dulbecco's Modified Eagle Medium |
| E2 | 17β-Estradiol |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| GSH | Glutathione |
| GTTR | Gentamicin tagged with Texas-Red |
| $H_A$ | Alternative Hypothesis |
| HC | Hair cells |
| $H_O$ | Null Hypothesis |
| IHC | Inner hair cells |
| IL-6 | Interleukin-6 |
| IL-1β | Interleukin-1β |
| JNKs | c-Jun N-terminal kinases |
| LPS | Lipopolysaccharide |
| M1 | Pro-inflammatory macrophages |
| M2 | Anti-inflammatory macrophages |
| MET | Mechano- electrical transduction |
| MLC | Microglia-like cell |
| hMSC | Human mesenchymal cells |
| Nf-kB | Nuclear Factor Kappa B |
| OHC | Outer Hair Cell |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| PTA | Pure-Tone Audiometry |
| ROS | Reactive Oxygen Species |
| SEM | Scanning electron microscope |
| SPL | Sound pressure level |
| TBS | Tris Buffered Saline |
| TEM | Transmission electron microscope |
| TGF-β | Transforming growth factor beta |
| TGP | Total glucoside of Paeony |
| TNF- α | Tumor necrosis factor- α |
| TRPV1 | Transient receptor potential cation channel subfamily V membrane 1 |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| cVEMP | Cervical vestibular evoked myogenic potentials |
| VsEP | Short-latency vestibular evoked potentials |

# Chapter 1: Introduction

## 1.1) The cochlea and cochlear hair cells

The cochlea is a spiral fluid-filled structure located in the inner ear. It contains hair cells (HCs) which are named after the small hair-like structures (stereo-cilia) present in their apical membrane. The cochlear HCs are located in the organ of Corti which rests on top of the basilar membrane (BM) and is covered superiorly by the tectorial membrane (Fig 1)

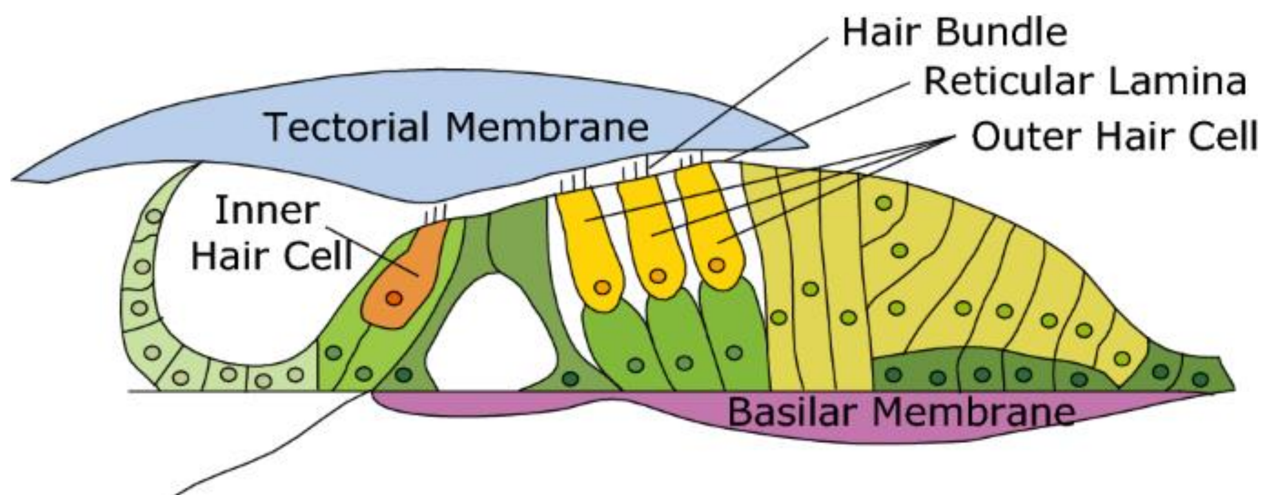



**Figure 1**. Anatomy of the organ of Corti shown in the coronal plane. Figure from Nankali, A., Wang, Y., Strimbu, C. E., Olson, E. S., & Grosh, K. (2020). A role for tectorial membrane mechanics in activating the cochlear amplifier. *Scientific reports, 10*(1), 1-15.

After traversing through the outer and middle ear, sound waves enter the inner ear via the oval window which results in vibration of the basilar membrane. The vibration of the basilar membrane creates a shearing motion between the stereocilia of the HCs and the tectorial

membrane. This causes the ion channels on the HC membrane to open, which ultimately results in HC depolarization. As a result of HC depolarization, neurotransmitters are released into the synaptic cleft between the HCs and cochlear afferent fibers. This generates electrical signals in the cochlear afferent fibers which are sent to the brainstem cochlear nucleus via the vestibulocochlear nerve (Carricondo and Romero‐Gómez, 2019; Fettiplace, 2011).

High-frequency sound waves vibrate the basilar membrane towards the base region of the cochlea while low-frequency sound waves vibrate the basilar membrane towards the apical region. Hence, different frequencies of sound waves cause the hair cells in different regions of the cochlea to move against the tectorial membrane. This allows the HCs from different cochlear regions (apex, middle, base) to be activated according to the different frequencies of the sound waves such that high-frequency sound waves can activate hair cells towards the base region of the cochlea while low-frequency sound waves can activate the hair cells towards the apex region. The signals sent from different cochlear regions connect to the brain in different parts of the auditory cortex. This enables the brain to distinguish the sound waves according to their frequency (Li et al., 2021).

There are two types of HCs in the cochlea: inner hair cells (IHCs) and outer hair cells (OHCs). The inner hair cells (IHC) are organized in a single row while outer hair cells (OHC) are organized in three rows along the cochlea (Figure 3).

While IHCs are involved in sound transduction as described above, the OHCs amplify the maximum vibration caused by the sound waves on the basilar membrane, thus helping the IHCs to detect low sound intensity (Ashmore, 2008).

In summary, the cochlea contains HCs which transduce the sound waves into signals that can be perceived by the brain and the cochlea plays a major role in distinguishing sound waves according to their frequency. Hence, the cochlea is crucial for the perception of sound.

### 1.2) The vestibular system and the utricle

The vestibular system is a complex set of structures located in the inner ear. Similar to the cochlea, the vestibular system also contains HCs in the three organs of the vestibular system: the 3 semi-circular canals (horizontal, anterior and posterior), the utricle, and the saccule. The HCs in these organs detect acceleration of the head in rotational, linear horizontal, and vertical planes respectively, and relay the information via the vestibulocochlear nerve to the vestibular nucleus within the brainstem. The vestibular nucleus in the brainstem interprets the afferent nerve signals carried by the vestibulocochlear nerve and combines the peripheral signals from different parts of the body to elicit head, eye, and body motor responses that are necessary for the maintenance of balance and orientation. Hence, the vestibular system is crucial for the maintenance of balance, vision and spatial orientation (Casale et al., 2018; Khan and Chang, 2013).

The utricle contains a sensory epithelium known as utricular macula upon which the utricular HCs reside. The utricular macula is covered by the otolithic membrane which is made up of gelatinous substance and embedded with calcium carbonate crystals (otoconia). Changes in linear motion of the head along the horizontal axis results in a shearing motion between the hair cells on the sensory epithelium and otolithic membrane. This causes the opening of non-specific mechano-transduction channels which then allows the entry of K+ ions into the utricular HCs. This event results in the depolarization of the utricular HC membrane and subsequent release of neurotransmitters into the synaptic cleft between the utricular HC and vestibular afferent

neurons. This in turn leads to the generation of electrical activity in the vestibulocochlear nerve. The electrical signals then travel to the ipsilateral cerebellum and vestibular nuclei in the brain and in this way, information regarding the change of acceleration in the horizontal axis is detected by the utricle and relayed to CNS (Meza, 2008).

### 1.3) Aminoglycosides and ototoxicity

Aminoglycosides are antibiotics used to treat gram-negative bacterial infections such as cystic fibrosis and life-threatening conditions such as sepsis. During 1943, the first aminoglycoside, streptomycin, was discovered and it was the only drug to effectively treat tuberculosis at that time (Waksman, 1953). Soon after, Hinshaw and Feldman (1945) revealed the ototoxic and nephrotoxic side effects of streptomycin. While the nephrotoxicity caused by aminoglycosides is generally reversible (Hock and Anderson, 1995), the ototoxicity caused by aminoglycosides can be permanent (Greenwood, 1959).

Ototoxicity refers to the ability to damage sensory hair cells in the inner ear structures, particularly the vestibular system and the cochlea. Low dose and in some cases, even a single dose of aminoglycoside can result in aminoglycoside-induced ototoxicity. Hence, therapeutic drug monitoring of aminoglycoside is unlikely to prevent the ototoxic effects of aminoglycosides as there is no safe dose of aminoglycoside that can ensure the lack of aminoglycoside-induced ototoxicity after its administration (Black et al., 2004; Halmagyi et al., 1994; Roland, 2003). Aminoglycosides such as neomycin, kanamycin and, amikacin, preferentially damage the hair cells in the cochlea while gentamicin and tobramycin preferentially damage the hair cells in the vestibular system (Matz, 1993). Damage to the cochlear hair cells can result in permanent hearing loss and tinnitus while damage to vestibular hair cells can result in vestibular system

disorders such as dizziness, loss of balance, and vertigo (O'neil, 2008). Previous literature has estimated that aminoglycoside-induced vestibular damage can result in vestibular disorders in approximately 15% of the patients undergoing aminoglycoside treatment (Fee, 1980), while aminoglycoside-induced cochlear damage can cause permanent hearing loss in about 10% of the patients receiving aminoglycoside treatment (Laurell, 2019).

Genetic predisposition that is, the mutation of mitochondrial gene (m1555 A→G) is one of the most well-known risk factors of aminoglycoside-induced ototoxicity (Fischel-Ghodsian et al., 1997; Xie et al., 2011). In addition, previous studies have suggested that both increasing age and decreasing liver function can also increase the risk of aminoglycoside-induced ototoxicity (Al-Malky et al., 2015; Gatell et al., 1987; Moore et al., 1984). Therefore, people with mitochondrial gene mutation, increased age and liver dysfunction can be at increased risk of aminoglycoside-induced ototoxicity.

Cochlear and vestibular disorders can have devastating effects on the quality of life of patients receiving aminoglycoside treatment. Aminoglycoside-induced cochlear damage and subsequent development of hearing loss can seriously impair an individual's ability to communicate with other people. This impairment can negatively affect the patient's socializing ability, employment prospects, self-esteem and general well-being. A study by (Ruben, 2000) estimated that the unemployment rate for people with hearing loss was 11% higher when compared to the unemployment rate for people with no disability (36% versus 25%). Furthermore, a study by (Tambs, 2004) found a significant association between hearing loss and reduction in mental health ratings, particularly in young and middle-aged participants. Similarly, a study by (Vogel et al., 2014) found that tinnitus, another symptom of aminoglycoside-induced

cochlear damage, could increase the risk of development of anxiety, depression and even suicidal thoughts. Thus, the aminoglycoside-induced cochlear damage can lead to permanent hearing loss and/or tinnitus and these symptoms can affect a patient's employment and mental well-being.

Vestibular disorders can place severe physical limitations and financial burden on patients since an individual suffering from vestibular disorders may be unable to drive vehicles or operate heavy machinery (Ariano et al., 2008). A retrospective study by (Black et al., 2004) found that, out of 33 patients receiving gentamicin treatment, none of the patients could retain their pre-morbid occupation following gentamicin-induced vestibular disorders. Moreover, 32 of the 33 patients had to depend on social and disability grants for daily subsistence. These findings clearly demonstrate the extent to which aminoglycoside-induced vestibular disorders can degrade the financial status of patients. Besides physical limitations and subsequent financial burden, aminoglycoside-induced vestibular disorders can also have a debilitating effect on a patient's cognitive ability and psychology. Vestibular disorders can lead to hippocampal atrophy which can then result in cognitive decline and behavioral changes leading to an increased risk of development of anxiety and depression (Smith et al., 2005). Thus, aminoglycoside treatment can cause vestibular disorders which can eventually lead to the decline of the patient's physical and mental capability, and place them under a substantial financial burden while also having negative effects on their physiological well-being.

During the 1980s, 3$^{rd}$ generation cephalosporins temporarily gained favour over aminoglycosides for the treatment of bacterial infections since they had a relatively higher efficacy but lower toxicity compared to aminoglycosides. However, it was found that bacteria could develop rapid tolerance to cephalosporins and therefore the interest in aminoglycosides was renewed (Krause et al., 2016). Hence, despite their permanent ototoxic effects,

aminoglycosides are still one of the most commonly prescribed antibiotics to this day as alternatives are still lacking (Grohskopf et al., 2005). In developed nations, aminoglycoside prescriptions are usually limited to the treatment of severe infections such as multi-drug resistant tuberculosis (Caminero et al., 2010). Meanwhile, in developing nations, aminoglycoside use is much more frequent due to their low cost, and aminoglycosides are prescribed for less severe bacterial infections such as bronchitis (Schacht, 1993). Globally, aminoglycosides are used to treat infants suspected or proven to be suffering from sepsis as sepsis is often life-threatening and the benefit of aminoglycosides outweighs the cost/adverse effects in such situations (Pacifici, 2009).

### 1.4) Mechanisms behind aminoglycoside-induced ototoxicity

Although previous studies have implied various mechanisms of action behind aminoglycoside-induced ototoxicity, the precise mechanism is not yet clear. The mechano-electrical transduction (MET) channels are located near the tips of stereocilia in hair cells and are responsible for sensory transduction (Qiu and Müller, 2018). Previous literature has implicated the involvement of MET channels for aminoglycoside uptake into the hair cells (Gale et al., 2001). Alharazneh et al. (2011) found that MET channel blockers significantly reduced the uptake of gentamicin tagged with Texas-Red (GTTR) in both, inner and outer hair cells. Hence the findings from Alharazneh et al. (2011) supported the theory that MET channels are involved in the uptake of aminoglycosides into hair cells. Like the MET channels, a cation channel known as transient receptor potential cation channel subfamily V member 1 (TRPV1) was found to facilitate the uptake of gentamicin into hair cells in Jiang et al. (2019)’s study. Additionally, TRPV1 was found to exacerbate gentamicin-induced cochleotoxicty during lipopolysaccharide

(LPS) stimulation by increasing the gentamicin uptake (Jiang et al., 2019). As LPS's are endotoxins produced by gram-negative bacteria and aminoglycosides are prescribed to patients infected with gram-negative bacteria, this result suggests that under clinical conditions, TRPV1 channels may play an important role in exacerbating the toxicity of aminoglycosides.

Once aminoglycosides penetrate the cell, they bind to their intracellular target which is the small subunit of the ribosome. They can bind to the ribosomes of both bacterial cells and mammalian cochlear hair cells. In the hair cells, aminoglycosides primarily bind to mitochondrial ribosomes (Hobbie et al., 2008). Previous literature has suggested that aminoglycoside ototoxicity is a result of aminoglycosides binding to human mitochondrial ribosomes (Greber et al., 2015; Guan et al., 2000; Hobbie et al., 2008). Evidence for this theory comes from early studies which found that certain mutations in mitochondria could make patients hypersensitive to aminoglycoside-induced ototoxicity such that in some cases, even a single dose of aminoglycosides could cause permanent hearing loss (Estivill et al., 1998; Hutchin et al., 1993; Prezant et al., 1993).

Once bound to the ribosomes in eukaryotic mitochondria, aminoglycosides can cause impairment of RNA translation and inhibition of protein synthesis within mitochondria (Hobbie et al., 2008; Prezant et al., 1993). Previous literature has suggested that the inhibition of protein synthesis leads to a decrease in ATP which eventually leads to the disintegration of the mitochondrial membrane, the release of cytochrome C, and apoptosis (Guan et al., 2000).Desa et al. (2018) also showed the correlation between change in mitochondrial function (such as mitochondrial membrane potential loss) and reactive oxygen species (ROS) production, during aminoglycoside-induced hair cell damage. Hence, aminoglycoside binding to mitochondrial ribosomes might cause mitochondrial dysfunction which might promote the ROS formation and

oxidative stress. Oxidative stress can result in lipid peroxidation, protein carboxylation, and nitrosylation, causing tissue damage and inflammation (Garcia-Alcantara et al., 2018).

An early study by Pullan et al. (1992) suggested that AGs such as neomycin can act as an agonist on the polyamine binding site of the NMDA receptor. Moreover, an experiment conducted by Basile et al. (1996) showed that NMDA receptor antagonists could attenuate both aminoglycoside-induced hair cell death and hearing loss. Additionally, a study by Segal et al. (1999) demonstrated that neomycin treatment could cause excitotoxicity in the striatum, a part of the central nervous system containing high NMDA receptor density. Hence there is evidence that aminoglycosides can cause excitotoxicity in the hair cells by acting as agonists at the polyamine site on NMDA receptors.

Recent literature has implicated the involvement of inflammation during aminoglycoside-induced hair cell death. Garcia-Alcantara et al. (2018) used IBA1 staining to detect a type of cell involved during inflammation, i.e., the activated microglia-like cell (MLC). They found that kanamycin and furosemide administration significantly increased the number of IBA1 positive cells which indicated increased recruitment of MLCs and therefore, increased inflammation. Furthermore, they also found the upregulation of pro-inflammatory and anti-inflammatory cytokine expression in the organ of Corti following aminoglycoside (kanamycin+ furosemide) treatment compared to the control group. Additionally, using the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, they found a significant increase in apoptotic cells in the (kanamycin+ furosemide) treatment group compared to the control group, indicating that inflammation was accompanied by cellular apoptosis in hair cells of the inner ear.

Likewise, another study by Sun et al. (2015) found that the mRNA and protein expression levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and

tumor necrosis factor- α (TNF- α), increased significantly in the neomycin treatment group compared to the control group, at day 1, 2, and 3. Furthermore, their results suggested that aminoglycosides could upregulate the expression of the cytokine ligand, fractalkine (CX3CL1), which could increase MLC activation and lead to an increase in pro-inflammatory cytokine (IL-1, IL-6, and TNF- α) expression. This increase in pro-inflammatory cytokine expression could then eventually cause inflammation and then cell death via apoptosis. The studies by Sun et al. (2015) and Garcia-Alcantara et al. (2018) provide evidence that aminoglycosides can induce inflammation in the inner ear. Furthermore, the results from these studies suggest that aminoglycoside-induced inflammation is one of the mechanisms behind aminoglycoside-induced hair cell death.

### 1.5) Gentamicin-induced ototoxicity

Gentamicin is a broad-spectrum aminoglycoside that was introduced as early as 1963. However, it is still one of the most commonly used aminoglycosides since it is widely available, highly efficacious, and inexpensive. Because of its wide clinical use, exposure to gentamicin is one of the most common causes of bilateral vestibular dysfunction (Black et al., 2004). Although the ototoxicity of gentamicin was investigated by numerous studies throughout the late 20$^{th}$ century (Aran et al., 1982; Bagger-Sjöbäck et al., 1990; Kotecha and Richardson, 1994; Marais and Rutka, 1998), the interest in gentamicin-induced toxicity has continued well into the early 21$^{st}$ century. A study by Nakamagoe et al. (2010) evaluated the effects of an anti-apoptotic agent, 17β-Estradiol (E2), on gentamicin-induced cell death in the cochlear explants of Sprague-Dawley rats (postnatal day 3-5). They treated the explants with gentamicin (0.1 mM), or gentamicin (0.1 mM) and E2 (1,10,100 and 1000 nM), for 48 hours and stained the explants for phalloidin. Phalloidin is a specific marker for cellular F-actin that labels the cuticular plate and

stereocilia of the hair cells. After staining the hair cells with phalloidin, they used fluorescence microscopy to view the explants and counted the hair cells. Compared to the control explants, the gentamicin (0.1 mM) treated explants exhibited 70 % OHC and 10% IHC loss in the basal turn of the cochlea and 20% OHC and no IHC loss in the apical turn of the cochlea. They hypothesized that greater HC loss would be observed in the apical coil if gentamicin exposure of higher concentrations was used. Nonetheless, their results suggest that cochlear damage by gentamicin (0.1 mM) treatment for 48 hours is mainly limited to loss of basilar OHCs. Since high-frequency sounds are detected by HCs in the basilar region of the cochlea, from this result it can be inferred that treatment with gentamicin (0.1 mM) is likely to result in the loss of hearing of high-frequency sounds. Nakamagoe et al. (2010) also found that the E2 treatment significantly decreased the ratio of OHC, but not IHC loss. This suggests that E2 could protect OHCs from gentamicin-induced damage but not the IHCs. They used TUNEL staining to label the apoptotic cells and after comparing the % TUNEL labeling between gentamicin (0.1 mM) treated HCs and gentamicin (0.1 mM) + E2 (100 nM) treated HCs, they deduced that E2 significantly decreased the TUNEL-staining in the HCs. Furthermore, they also found that the labeling for an apoptotic pathway, the c-Jun N-terminal kinases (JNKs), was significantly lower in gentamicin (0.1 mM) + E2(100 nM) treated HCs when compared to gentamicin (100 μM) treated HCs. In summary, the results from this study indicate that gentamicin (0.1m M) damaged the OHCs in the basal region of the cochlea by activating the JNK pathway and promoting cellular apoptosis while E2 attenuated the gentamicin-induced apoptosis in the OHCs by inhibiting the JNK pathway and downregulating HC apoptosis.

A study by Bramhall et al. (2014) garnered much attention since the result from this study suggests that Lgr-5 expressing supporting cells might transdifferentiate into hair cells to replace

the hair cells damaged during aminoglycoside treatment. The findings from this study were interesting as hair cell regeneration was thought to occur only in avian species and not in mammals. However, the findings from this study imply that although not as successful as in the avian species, the supporting cells attempt to regenerate the aminoglycoside-damaged hair cells in mammals. In this study, the cochlear explants from genetically modified mice were treated with gentamicin (0.05 mM) for 16 hours and the explants were cultured in drug-free media for additional 72 hours. The explants were then stained with MYO7A to identify the HCs and hair cell counts were obtained per 100 µm under a Leica TCS SP5 confocal microscope. They reported 9%, 42% and 82% OHC loss in the apical, middle and basal region of the cochlea, respectively. They also mentioned that they observed "limited" IHC loss but did not quantify the IHC loss clearly.

From the in-vitro studies discussed above, it can be deduced that the damage induced by gentamicin (0.05 – 0.1 mM) is mainly limited to basal OHCs. However, as demonstrated by Kotecha and Richardson (1994), gentamicin treatment at an increased dose (0.5 mM and 1 mM) can damage hair cells at both the apical and basilar region of the cochlea, as early as 1 hour after exposure.

Another in-vitro study by Kim et al. (2009) investigated the role of oxidation and apoptosis during gentamicin-induced vestibular damage in 2-4 Sprague-Dawley rats and evaluated the anti-oxidative effects of melatonin against gentamicin-induced vestibular damage. After isolating the utricles from the animals, they cultured the utricular explants in DMEM media containing gentamicin (1 mM) or gentamicin (1 mM) + melatonin (0.01, 0.05 or 0.1 mM), for 1, 4 and 7 days. They stained the vestibular HCs with phalloidin- fluorescein isothiocyanate and observed the explant under a confocal laser scanning microscope (CLSM, ZEISS510 META, Gottingen,

Germany). They counted 292.8 ± 21.8, 292.0 ± 10.1 and 288.4 ±8.0 utricular HCs at the 1st, 4th and 7th day, respectively, in the control utricular explants. It was found that the gentamicin treatment drastically reduced the number of utricular hair cells to 48.2 ± 15.9 38.4 ± 8.1 and 19.8±11.1 (approximately 83%, 86 %, and 93% reduction in HC number) on the 1st, 4th and 7th day, respectively. This result suggests that gentamicin (1 mM) can cause extensive vestibular hair cell degeneration as early as 24 hours and the severity of gentamicin-induced vestibular hair cell damage increases with increasing duration of exposure to gentamicin.

Compared to gentamicin (1 mM) alone treated explants, gentamicin (1 mM) and melatonin (100 µM) treated explants exhibited a significant increase in the number of surviving HCs (68.6±19.5 (n = 5), 161.0±22.0 (n = 5), and 154.4±14.3 (n = 5) on the 1st, 4th, and 7th day, respectively. This result implies that melatonin can protect vestibular HCs from gentamicin-induced damage and a longer duration of melatonin exposure is more effective at protecting the vestibular hair cells from aminoglycoside-induced damage. Furthermore, they used H2DCFDA fluorescence activity to determine the extent of ROS generation and observed a weak ROS expression for gentamicin and all melatonin treatment groups. In contrast, they observed strong ROS expression for the gentamicin-only treatment group. Upon measuring the caspase-3 activity, they found that the gentamicin (1mM) only treatment group showed the strongest caspase-3 expression and melatonin treatment (0.05 mM) attenuated the activation of caspase-3. From these results, it can be inferred that the vestibular HCs were damaged by gentamicin via the induction of ROS production and the activation of a caspase-3 mediated apoptotic pathway and, the gentamicin-induced damage was attenuated by melatonin via reduction of gentamicin-induced ROS production and caspase-3 activation. In concurrence with the finding of this study, (Cunningham et al., 2002) implicated apoptosis as one of the mechanisms behind

aminoglycoside-induced vestibular hair cell death. They proposed that caspase-9 activation and subsequent caspase-3 activation mediates aminoglycoside-induced vestibular HC death.

| Article | Aminoglycoside | Animal | Vestibular damage | Cochlear damage | Concentration | Duration of treatment | Key Results |
|---|---|---|---|---|---|---|---|
| Nakamagoe et al., (2010) | Gentamicin | Sprague-Dawley rats ( p3-p5) | NA | 70 % OHC loss 10 % IHC loss in basal region, only 20% OHC loss in apical turn | 100 µM | 48 hrs | E2 protects HCs from GENT by deactivating apoptotic JNK pathway. |
| Bramhall et al., (2014) | Gentamicin | Genetically modified mice | NA | 9%, 42% and 82 % OHC loss in apical , middle and basal region | 50 µM | 16 hrs followed by 72 hours in drug free media | Lgr-5 expressing supporting cells may be involved in HC regeneration |
| Kim et al., (2009) | Gentamicin | Sprague-Dawley rat (p 2-4) | GENT 1 mM significantly reduced utricular HC density by | NA | 1 mM | 1,4, and 7 days | Gentamicin-induced damage was attenuated by melatonin |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | (approximately 83%, 86 %, and 93% reduction in HC number) on the 1st, 4th and 7th day, respectively. | | | | via reduction of gentamicin-induced ROS production and caspase-3 activation. |

**Table 1**. Summary of in-*vitro* studies exploring gentamicin-induced ototoxicity.

Early studies have pointed out that although gentamicin is not selectively vestibuloxic, it is more toxic to the vestibular system than it is to the cochlea (Marais and Rutka, 1998). Concurrent with the observations of gentamicin-induced vestibular toxicity in the early studies, findings from Selimoğlu et al. (2003) also highlighted the vestibular toxicity of gentamicin as they found that all the organs of vestibular system, that is, the cristae ampullare in the 3 semi-circular canals, utricle, and saccule, were severely damaged by gentamicin.

The studies of gentamicin-induced ototoxicity as discussed above (Section 1.5) could have evaluated and made comparison between the vestibular effects and the cochlear effects of gentamicin rather than limiting the evaluation of gentamicin-induced damage to either the cochlea or vestibular system. This could have provided more clarity to gentamicin's toxicity profile as the findings from studies such as Aran et al., (1982) indicate that gentamicin damages both the cochlea and vestibular system equally while Marais et al. (1998)'s findings suggest that gentamicin damages the vestibular system more than the cochlea. Meanwhile, findings from a

clinical study by Baggaer-Sjoback et al., (1990) have even suggested that gentamicin damages the cochlea more than the vestibular system. Hence, future studies could compare the effects of gentamicin on both the cochlea and vestibular system to clarify the ototoxic profile of gentamicin.

### 1.6) Amikacin-induced ototoxicity

Amikacin is a semi-synthetic aminoglycoside that was introduced in the late 1970s to overcome the problem of bacterial resistance against aminoglycosides (Jackson, 1983; Ramirez and Tolmasky, 2017). It is commonly used to treat infections such as non-tuberculous mycobacterial infections and gentamicin-resistant gram-negative bacterial infections (Kim et al., 2016). However, the problem of ototoxicity persisted with amikacin and its ototoxic effects were highlighted by a clinical study during the 1970s (Black et al., 1976). Upon evaluating the changes in cochlear morphology using a scanning electron microscope (SEM) and transmission electron microscope (TEM), Lenoir et al. (1999) reported that all OHCs and most IHCs were destroyed along the length of the cochlea of 9 day old Wistar rats that were treated with amikacin (500 mg/kg) for 7 days. The evidence of amikacin-induced cochlear damage was further supported by the findings from Ladrech et al. (2007)'s study. Similar to Lenoir et al. (1999), Ladrech et al. (2007) also used SEM and TEM to analyze the cochlear hair cells. They observed that after 7 days of amikacin (500 mg/kg) treatment, the middle cochlear turns from Wistar rats (9-16 days old) did not exhibit any surviving OHCs and showed only a few damaged IHCs. In addition to morphological damage, Ladrech et al. (2007) also evaluated macrophage density as a way to evaluate the inflammation caused by amikacin. They found that the macrophage density was nearly 20-fold higher in the amikacin treatment groups when compared to the control group.

This is one of the few studies that has evaluated inflammation as one of the mechanisms behind amikacin-induced cochlear toxicity and from their results, inflammation is likely to be involved during the amikacin-induced cochlear toxicity. Future studies could elucidate the molecular pathways involved in amikacin-induced inflammation by measuring the level of inflammatory cytokines since the increase in macrophage density typically leads to a change in inflammatory cytokine levels (Sun et al., 2015).

An *in-vitro* study by Kim et al. (2016) investigated the role of oxidation during amikacin-induced cochlear damage. They treated the cochlear explants obtained from institute of research mice (ICR) (Post-natal day 3) with amikacin (0.5, 1 and 2 mM ) for 48 hours. Explants treated with amikacin (2 mM) exhibited complete IHC and OHC loss in all regions of the cochlea. Compared to the control explants, the amikacin (1 mM) treated explants exhibited significant IHC loss (approximately 91%, 86%, and 91% IHC loss in the apical, middle and basilar region, respectively) and significant OHC loss (approximately 70%, 95%, and 100% OHC loss in the apical, middle and basilar region, respectively). Meanwhile, amikacin (0.5 mM) treated explants exhibited significant but reduced OHC loss (approximately 29%, 26%, and 91% OHC loss at the apical, middle, and basilar region, respectively) while the number of IHCs in amikacin (0.5 mM) treated explants were not significantly different from the control explants. These results suggest that damage by amikacin (0.5 mM) treatment is mainly limited to basilar OHCs while treatment with amikacin at a concentration of 1 (mM) or higher can result in severe IHC and OHC loss in all regions of the cochlea.

MitoSox-Red is an indicator for the specific detection of ROS (mitochondrial superoxide). Upon using the MitoSox-Red stain to evaluate ROS production, Kim et al. (2016) found that the number of MitoSox-Red positive IHCs and OHCs had significantly increased in the apical,

middle, and the basal region of the cochlea. Furthermore, they also found that treatment with an anti-oxidative agent, galangin (0.01 mM) in addition to amikacin (1 mM), significantly reduced the MitoSox-Red positive OHCs and IHCs in all regions of the cochlea. Moreover, the galangin (0.01 mM) and amikacin (1 mM) treatment significantly increased IHCs and OHCs survival in all regions of the cochlea. These results suggest that amikacin (1 mM) damaged the IHCs and OHCs of the cochlea by promoting ROS production and galangin protected the IHCs and OHCs from amikacin-induced damage by decreasing ROS level in the cochlea. However, without the assessment of vestibular damage, this study is limited to the investigation of only cochlear toxicity caused by amikacin.

| Article | Aminoglycoside | Animal | Vestibular damage | Cochlear damage | Concentration | Duration of treatment | Key Results |
|---|---|---|---|---|---|---|---|
| Ladrech et al., (2007) | Amikacin | Wistar rats (P 9) | NA | Macrophage density increased by 20-fold as early as 1w post-treatment, clearly present at 3w post-treatment. | 500 mg/kg | 7 days Explants also analyzed after 3 or 5-10 weeks post-treatment. | AMK treatment caused inflammation in the cochlea. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Kim et al., (2016) | Amikacin | ICR rats (P3) | NA | IHC and OHC extensively damaged by 1 & 2 mM AMK, only OHC damaged by 0.5 mM AMK. | 0.5, 1, 2 mM | 48 hrs | Galangin prevents ROS formation and protects against AMK-induced ototoxicity |

**Table 2**. Summary of in-*vitro*/*in-vivo* studies exploring amikacin-induced ototoxicity.

In contrast to gentamicin, amikacin is believed to damage the cochlea more than the vestibular system (Javadi et al., 2011; Yian and Xiaodong, 1995). A clinical study by Black et al. (1976) observed the incidence of ototoxicity in 77 patients in patients infected with a leukopenic (58), gentamicin-resistant organism (11), or suffering from cystic fibrosis (8) and receiving amikacin treatment courses at a dosage of > 7.5 mg/kg every 8 hours. Using pure tone audiometric tests, they found that the patients developed symptoms of cochlear toxicity such as the development of high-frequency hearing loss (n = 13) and tinnitus (n = 3). However, no patients reported signs of vestibular toxicity (i.e., dizziness and vertigo). They also found that therapy for greater than 10 days or more with amikacin (15g) was significantly associated with hearing impairment. Thus, the results from this clinical trial supports the theory that amikacin is more toxic to the cochlea than it is to the vestibular system. This theory is further supported by a cross-sectional study conducted by Javadi et al. (2011). They assessed the incidence of amikacin-induced ototoxicity in 41 multi-drug resistant tuberculosis patients receiving intravenous

amikacin (500 mg daily as 30 minutes IV infusion over 30 minutes) over 45.07 +/- 27.67 days. Using baseline Pure-Tone Audiometry (PTA), they found that 29 of the 41 patients (70%) had developed symptoms of hearing loss.

Although no patients reported vestibular dysfunction, these data are based only on questionnaires asking people if they experienced signs of vestibular toxicity. As no equipment was used to specifically detect vestibular disorders by either Black et al. (1976) or Javadi et al. (2011), the vestibular damage of amikacin might have been understated by these studies. In contrast, a study by Chen et al. (2013) tested for both cochlear and vestibular disorders using American Speech-Language-Hearing Association (ASHA) stratification and caloric-evoked nystagmus and cervical vestibular evoked myogenic potentials (cVEMP), respectively, in twenty-three cancer patients aged 3-8 years who were receiving amikacin for the treatment of febrile neutropenia or gram-negative infections. The patients received amikacin therapy for at least 15 days, and received at least 6,000 mg of amikacin cumulatively. Chen et al. (2013) detected hearing loss in three out of 23 subjects (13%) and vestibular disorders in four out of 23 subjects (17%). Hence, after testing for both vestibular and cochlear disorders as opposed to relying on questionnaires for detection of vestibular disorders, they found that vestibular disorders were slightly higher than cochlear disorders in patients receiving amikacin treatment. Furthermore, an in vitro study by Yian and Xiaodong (1995) compared the effects of amikacin, gentamicin, and normal saline on the vestibular organs and found that although amikacin was less vestibulotoxic than gentamicin, amikacin damaged the vestibular system with different degrees of severity, with the crista ampullarae showing the greatest signs of amikacin-induced damage followed by the utricle and the saccule. Hence, it might be worthwhile to compare the vestibular and cochlear toxicity of amikacin, especially since the vestibular toxicity of amikacin

is relatively underexplored. The use of vestibular and cochlear explant cultures might be useful for this purpose as previous studies have pointed out that diagnosis of vestibular dysfunction is relatively challenging compared to diagnosis of cochlear malfunction (Freeman et al., 2001) and this might result in understating the vestibular damage *in vivo* or in human clinical studies.

### 1.7) Critical analysis of aminoglycoside ototoxicity literature

Currently, there is a lack of studies that evaluate and compare aminoglycoside-induced cochlear and vestibular toxicity. Experimental protocols such as the duration of exposure to aminoglycosides, aminoglycoside concentration, and method of cochlear/vestibular toxicity evaluation are often different between a study evaluating cochlear toxicity and another study investigating the vestibular toxicity induced by an aminoglycoside. This makes it difficult to compare the vestibular and cochlear toxicity of the aminoglycoside as the difference in experimental protocols is likely to affect the comparison between cochlear and vestibular toxicity. Hence, future studies should compare the aminoglycoside's toxicity in both the cochlea and the vestibular system using the same experimental protocol in order to obtain a more accurate and complete ototoxic profile of aminoglycosides. Furthermore, evaluation of aminoglycoside's effects on both the cochlea and the vestibular system would save animal lives as the data relating to both cochlear and vestibular toxicity can be obtained from the same animal.

Although early studies such as Aran et al. (1982) have evaluated and compared the cochlear and vestibular toxicity of early aminoglycosides such gentamicin, tobramycin and dibekacin, similar comparisons of vestibular and cochlear toxicity between aminoglycosides are very few. Moreover, studies that compare the toxicity of aminoglycosides using the ex vivo utricular

culture system are extremely rare. Hence there is a need for the development of the ex vivo utricular culture system as a method to directly compare the toxicity profiles of existing and ultimately any new aminoglycosides.

**1.8) Total glucoside of paeony**

Paeoniae Radix Alba (PRA, called *baishao* in China), is the root of a traditional Chinese medicinal plant, *Paeonia lactiflora* Pall. PRA has been shown to have rich medicinal properties and has been used in China for over 2000 years for the nourishment of blood, regulation of menstruation, pain alleviation, and treatment of giddiness and fever. Total glucoside of Paeony (TGP) is considered to be the main bioactive ingredient of PRA. Previous studies have observed that TGP can exhibit anti-inflammatory, antioxidative, and analgesic activity without evident toxicity (He and Dai, 2011; Zhang and Dai, 2012). Furthermore, recent literature has found TGP to be a promising treatment for autoimmune diseases, such as rheumatoid arthritis (RA) (Luo et al., 2013) and psoriasis (Zheng et al., 2019). It has also been found that TGP can downregulate the expression of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and upregulate the expression of anti-inflammatory cytokines (IL-10 and TGF-β) (Cao et al., 2011; Lin et al., 2017).

Pro-inflammatory signaling and oxidation can increase the translocation of NF-kB transcription to the nucleus. The NF-kB transcription factors can then induce the transcription of pro-inflammatory mediators. (Naveed et al., 2018) observed that TGP can block the NF-kB signaling pathway by blocking the NF-kB translocation into the nucleus. This finding was supported by Gu et al. (2017)'s study which found that Paeoniflorin, the main component of

TGP (Figure 2), could inhibit the NF-KB pathway and exhibit anti-inflammatory effects on TNBS-induced ulcerative colitis.

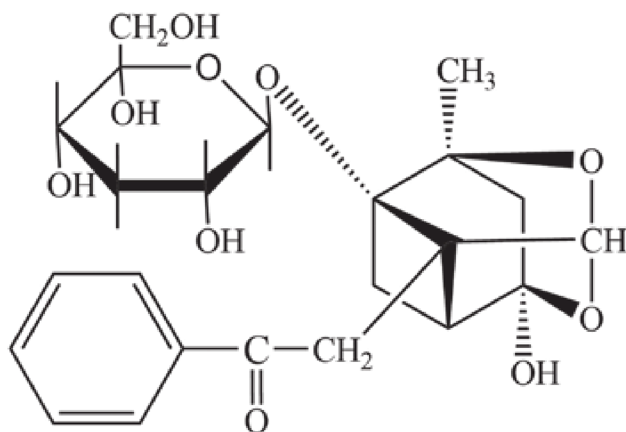



**Figure 2**. Chemical structure of Paeoniflorin. Figure from Ye, S., Mao, B., Yang, L., Fu, W., & Hou, J. (2016). Thrombosis recanalization by paeoniflorin through the upregulation of urokinase-type plasminogen activator via the MAPK signaling pathway. *Molecular Medicine Reports, 13*(6), 4593-4598.

Overall, these studies provide evidence for the anti-inflammatory effects of TGP. Since inflammation is one of the mechanisms by which aminoglycosides can cause hair cell death, TGP's anti-inflammatory effects might ameliorate aminoglycoside-induced hair cell death in the cochlea. To the best of our knowledge, the otoprotective potential of TGP has not yet been investigated.

Previous studies have suggested that various agents which reduce the downstream effects of AG such as: antioxidant agents (for example, melatonin) (Kim et al., 2009), ROS scavenger (for example galangin) (Kim et al., 2016), cell death blockers (for example estradiol) (Nakamagoe et

al., 2010), and upstream effects of AG such as bizbenzoquinoline that prevent AG entry into hair cells (Kruger et al., 2016), can potentially reduce aminoglycoside-induced toxicity. Despite these findings, there is no otoprotective medication available for the prevention or treatment of aminoglycoside-induced ototoxicity. Moreover, modulation of inflammation after aminoglycoside exposure has not been explored as a strategy to minimize aminoglycoside-induced ototoxicity. Hence, it might be worthwhile to by assess the potential otoprotective effects of anti-inflammatory agents such as TGP on both, cochlear and vestibular explants.

# Aims

1. To compare and establish the cochlear and vestibular toxicity profile for different aminoglycosides (gentamicin and amikacin).

2. To investigate if TGP can protect cochlear and vestibular hair cells from aminoglycoside-induced hair cell death.

# Chapter 2: Methods

## 2.1) Preparation for the dissection of cochlear and vestibular explants

Petri dishes, dissection tools, glass vials, and 13 mm coverslips were placed into an autoclave machine (Tuttnauer, 5075EL) and autoclaved a day before dissection. Phosphate-buffered saline (PBS) (0.01M, Gibco, 2779857) and Dulbecco's Modified Eagle Medium (DMEM) media (Gibco, 2200905) were filtered and transferred into the autoclaved glass vials under sterile conditions. Culture media containing DMEM, L-Glutamine (1%), Fetal bovine serum (FBS) (10%), and 30 µg/ml penicillin was prepared and transferred into autoclaved glass vials under sterile conditions.

The coverslips were placed into individual glass wells and coated with the collagen mixture to ensure that the cochlear and utricular explants would adhere to the coverslip and also to prevent the explant from folding on to itself. Briefly, collagen gel was prepared by mixing rat tail collagen I (9 mg/ml, Corning, 0090001) with DMEM, 1 M NaOH, and MiliQ water. After that, 20-30 µl of the collagen gel mixture was transferred and spread evenly over the coverslips using pipette tips. The coverslips coated with collagen gel mixture were placed in an incubator (Thermo Scientific, HERAcell 240i) and incubated for 2 hours at 37 °C.

## 2.2) Dissection of cochlear and vestibular explants

All animal procedure was approved in AUP-19-205 by the University of Otago Ethics Committee in accordance with New Zealand Animal Welfare act 1999. The dissection of the cochlear and vestibular explants as mentioned in this section (section 2.2) was performed by Associate Professor Yiwen Zheng.

Wistar rat pups (postnatal day 3 - 4) were euthanized by decapitation. Following this, the rat heads were immediately transferred to a sterilized petri dish and sprayed with 70% ethanol and the skull was bisected using a no.22 scalpel. The brain was removed using a no.7 curved forceps and the following steps were performed under a dissection microscope ( Meji Techno CO.LTD, BM23278), using a pair of no.55 fine tip forceps (Fine Science Tools, Dumont #55, 111295-51). The inner ear was separated from the temporal bone and surrounding tissues and transferred to another petri dish filled with ice-cold filtered DMEM. The petri dish containing the inner ear was then placed on a bigger petri dish containing ice. This was done to prevent inner ear cell degeneration during further dissections. Following this step, the utricle was isolated from the inner ear and the otolithic membrane covering the utricular macula was gently removed. The isolated utricle was then transferred to a DMEM-filled Petri dish and stored on ice until the end of the dissection. After this, the cochlear capsule was separated from the remaining inner ear structure and decapisulated. Organ of Corti was carefully unwound from the modiolus bone and separated from the stria vascularis. The basilar membrane and tectorial membrane were then removed from the isolated organ of Corti. For simplicity, the isolated organ of Corti will be referred to as the “cochlear explant” and utricular macula will be referred to as “vestibular explant”.

**2.3) Culturing the cochlear and vestibular explants**

Approximately 100 µL DMEM media was pipetted into the glass well containing collagen covered coverslips. The vestibular and cochlear explants were carefully transferred into the glass wells using a micro-spoon spatula. The explants were carefully positioned on top of the gel and the DMEM was pipetted out from the glass wells. The glass wells containing the explants were placed inside a petri dish and 500 µl of culture media was transferred into each

glass wells under sterile conditions. The Petri dish containing the glass wells which in turn contained the explants was placed in a $CO_2$ incubator (Thermo Scientific, HERAcell 240i) and incubated at 37°C and 5% $CO_2$ for 24 h before the drug treatment.

**2.4) Drug treatment of cochlear and vestibular explants**

Gentamicin (143.28 mg) (Sigma-Aldrich, G1264) was dissolved in 10 mL DMEM to make gentamicin (30 mM) stock solution. Likewise, amikacin (175.68 mg) (Sigma-Aldrich, LRAC3726) was dissolved in 10 mL DMEM to make amikacin (30 mM) stock solution. Under sterile conditions, the stock gentamicin and amikacin solution (30 mM) were filtered using 0.22 µM filter (Millex GV, SLCV03388) and further diluted in DMEM media to make the drug solution with the desired concentration.

For the drug treatment, the culture media was carefully removed from the glass well containing the coverslip on which the explant was placed. The drug solution (500µL) was then gently added to the well, with care being taken not to make the explant float away from the coverslip. In this way, the cochlear and vestibular explants were treated with gentamicin (0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM), gentamicin (0.3 mM) + TGP (100 µg/ml), gentamicin (0.9 mM) + TGP (100 µg/ml), amikacin (0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM), amikacin (0.6 mM) + TGP (100 µg/ml), or they were left untreated (control group) and the explants were incubated at 37°C and 5% $CO_2$ for 24hr.

**2.5) Staining the cochlear and vestibular explants with Phalloidin**

Twenty-four hours after the drug treatment, the cochlear and vestibular explants were stained with phalloidin to label the actin filaments in the stereocilia of cochlear and utricular hair cells. Briefly, the cochlear and vestibular explants were fixed with 4% paraformaldehyde (PFA)

(Sigma-Aldrich, MKCJ7612) for 30 min and incubated in PBS and Tween-20 (Sigma, 038K00915) mixture (19:1) for 20 min. Tween-20 was used to promote the entry of phalloidin into the cochlear and utricular hair cell. The wells containing cochlear explants were washed with PBS three times for 5 minutes each. Following this, a dilutant was prepared by mixing Tris Buffered Saline (TBS) (1 ml), Bovine serum albumin (BSA) (10 mg), and Triton (5µL). The staining solution was then prepared by diluting Phalloidin-iFluor 488 reagent (Abcam, ab176753) in a 1:1000 ratio with the prepared dilutent. The staining solution was then added to wells with the explants and incubated at room temperature for 60 min.

**2.6) Securing the explants on microscope glass slides.**

The surface of the microscope slide was covered with a strip of Scotch magic tape. A square big enough to fit the coated coverslip was carved out of the tape. The coated coverslip containing the cochlear explant or vestibular explant was placed in the middle of the carved out square and mounting medium (slow fade gold antifade, Invitrogen, 2339807) was added to the explants. The explant was then covered with a square coverslip and sealed with nail polish. The microscope slides were then stored inside an opaque box to avoid the interaction of light particles with phalloidin stain and incubated overnight at 4°C.

**2.7) Viewing and counting the cochlear and utricular hair cells**

The cochlear and utricular hair cells were viewed under a fluorescent microscope (ZEISS AXON & NIKON N: Eclipse Upright Microscope) with an excitation wavelength of 493 nm and an emission wavelength of 517 nm. Images of cochlear explants were taken under ×20 magnification while images of utricular hair cells taken under ×40 magnification using a NIS-Element software.

In a single column of hair cell, there are approximately 1 IHC and 3 OHCs (Figure 3).

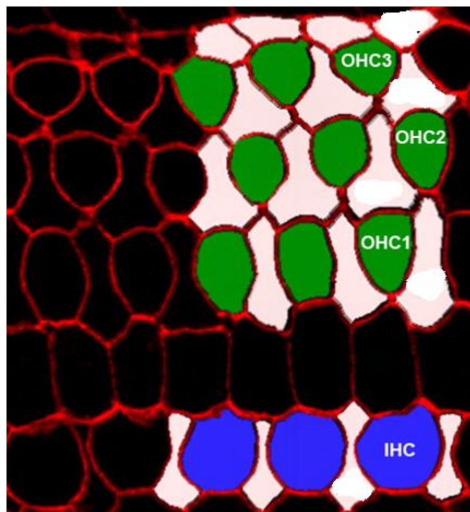



**Figure 3.** Confocal image of P0 mouse cochlea. IHCs (blue) are arranged in single row while OHCs (green) are arranged in three rows along the cochlea. Figure is reproduced from Etournay, R., Lepelletier, L., de Monvel, J. B., Michel, V., Cayet, N., Leibovici, M., . . . Petit, C. (2010). Cochlear outer hair cells undergo an apical circumference remodeling constrained by the hair bundle shape. *Development, 137*(8), 1373-1383.

The columns of hair cells are more orderly and apparent while viewing the cochlear explant under a fluorescent microscope (Figure 4). In the control group, we observed approximately 1 IHC and 3 OHC per hair cell column (Figure 4).

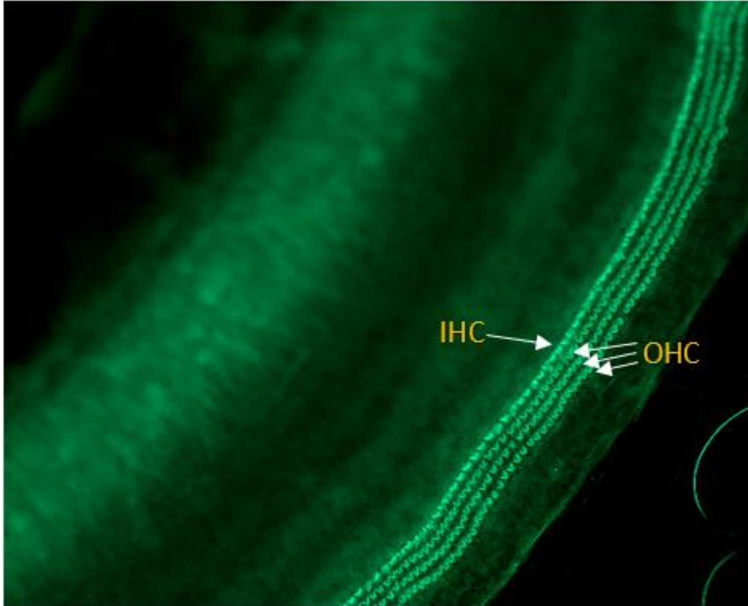



**Figure 4**) An image of a cochlear explant without any treatment, taken under a florescent microscope.

Therefore, after accounting for the stained/surviving IHCs and OHCs in a column of hair cells we could estimate missing hair cells in that column. For example, if we observed only 2 OHCs in a column of hair cell, this would indicate that 1 IHC and 1 OHC were missing from that particular column of the hair cells.

On average, we counted stained/surviving and missing IHC and OHCs through 35 HC columns, in 5 areas per cochlea. In each area, the IHC/OHC survival rate was calculated using the following formula:

$$\text{HC survival rate} = \frac{Number\ of\ surviving\ HC}{(Number\ of\ surviving\ HC + Number\ of\ missing\ HC)}$$

The IHC and OHC survival rate of all cochlear areas were averaged to calculate the average IHC and OHC survival rate for each cochlea. The average IHC and OHC survival rate of individual cochlear explants in the treatment group was then divided with the average IHC and OHC

survival rate of the control group and the result was multiplied by 100 to calculate the % control survival rate of the IHC and OHC.

### 2.8) Measurement of vestibular HC fluorescent intensity

For each utricular explants, four areas (0.07 $mm^2$) were chosen for the analysis if the HCs were not covered by otolithic membrane and did not show any signs of surgical trauma. The number of HCs were counted in each area and then averaged to represent the average number of utricular HCs/ 0.07 $mm^2$ in a utricular explant. The average numbers of uticular HCs/ 0.07 $mm^2$ of each utricle in the treatment groups was divided by that in the control group and multiplied by 100 to calculate the % control survival rate of the utricular HC.

### 2.9) Statistical Analysis

Levene's test was performed using SPSS software. All other statistical analysis was performed using Prism Graph-Pad 9 software. The normality and equal variance were checked for the data being analyzed and then the appropriate parametric or non-parametric test were chosen. The Shapiro Wilk test was used to check if the data in the analyses performed (that is, the regression, the unpaired t-tests, the one-way ANOVAs, and the two-way ANOVAs) were normally distributed. The normality of the residuals was reported unless specified otherwise. The Levene's test based on mean was used to check if the variances between the treatment groups included in the analyses were approximately equal. A dose-response curve was graphed with increasing aminoglycoside (gentamicin or amikacin) concentration on the X –axis and the average HC (IHC or OHC or utricular HC) survival (% control) ± SEM on the Y-axis. Regression analyses such as the simple linear regression or the non-linear regression were used to predict the value of the dependent variable Y (that is, the HC survival % control) from the value of the independent

variable X (that is, the aminoglycoside concentration) and the $TC_{50}$, the concentration of aminoglycosides required to reduce the HC survival rate by 50%, was calculated.

A two-way ANOVA was performed to find if the two independent variables: the type of aminoglycoside used (that is, gentamicin or amikacin) and the doses of aminoglycoside used (that is, the doses of gentamicin and/or amikacin), had any significant effect on the dependent variable that is, the HC survival (% of control). A significant aminoglycoside type effect would indicate that the HC survival between the gentamicin and amikacin-treated explants were significantly different ($H_A$) while a non-significant aminoglycoside type effect would indicate that the HC survival between the gentamicin and amikacin-treated explants were approximately equal ($H_O$).

Meanwhile. a significant aminoglycoside dose effect would indicate that the degree of HC survival between the explants treated with the different doses of gentamicin and/or amikacin were significantly different ($H_A$) while a non-significant aminoglycoside dose effect would indicate that the HC survival between the explants treated with the different doses of both gentamicin and amikacin were approximately equal ($H_O$). If the aminoglycoside type had a significant effect on the HC survival, a Bonferroni post-hoc analysis was performed to compare the differences in the HC survival between the individual gentamicin and amikacin treatment groups. Furthermore, separate one-way ANOVAs were performed to identify which aminoglycoside dose, that is, either the gentamicin doses or the amikacin doses or both the gentamicin and amikacin doses, had a significant effect on the HC survival.

The Shapiro-Wilk test tests the null hypothesis that the residuals of the data are normally distributed. Thus, if the p-value is greater than 0.05 in this test, then the null hypothesis is accepted and this result indicates that the residuals of the data are normally distributed. In contrast, if the p-value for the Shapiro-Wilk test is less than 0.05, this indicates that the data are not normally

distributed and non-parametric tests like Mann Whitney test should be used for comparison between two treatment groups since the Mann Whitney test does not assume that the data being compared follow a normal distribution (Nachar, 2008).

The Levene's test tests the null hypothesis that the groups compared have equal variance. Hence, if the p-value is greater than 0.05 in this test, then the null hypothesis is accepted and this result indicates that the variances between the groups being compared are approximately equal. However, if the p-value for the Levene's test is less than 0.05, this result indicates that the variances between the groups are significantly different in which case, the Welch test can be used for comparison between two treatment groups since the Welch test does not assume that the variances between the groups being compared are equal. However, the Welch test is sensitive to violation of the normality assumption so, this test should only be used for comparing two groups with unequal variances provided that the data in both groups are normally-distributed (Ahad and Yahaya, 2014).

Hence, for the comparison of HC survival data (% of control) between any two treatment groups, the non-parametric Mann Whitney test was used if the normality could not be assumed for either of the groups being compared. The Welch test was used for comparison between the treatment groups if the data in the treatment groups being compared were normally distributed but the variances between the treatment groups were found to be significantly different. Alternatively, an unpaired t-test was used to compare the difference between the treatment groups if the treatment groups being compared were normally distributed and the variances between the treatment groups were not significantly different. The statistical analysis for the comparison of the HC survival data (% of control) between any two treatment groups is summarised in the following figure (Figure 5).

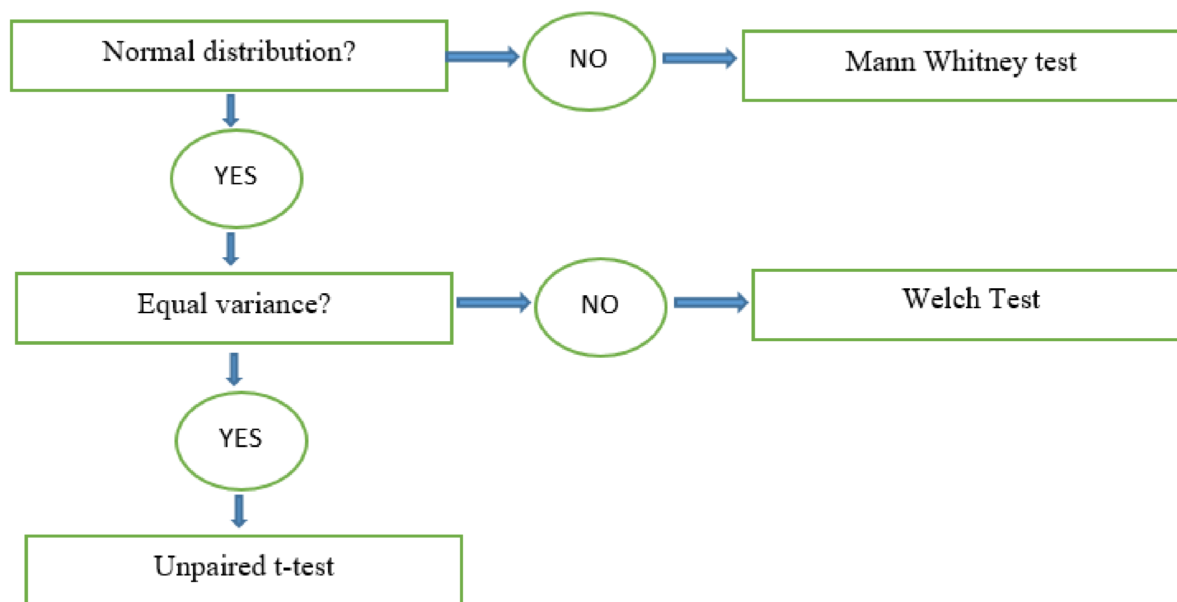



**Figure 5**. Summary of the statistical analysis for the comparison of the HC survival data (% of control) between any two treatment groups.

Likewise, a non-parametric Kruskal-Wallis test was used instead of the one-way ANOVA if normality could not be assumed for the data being analyzed and/or the variances between the treatment groups being compared in the one-way ANOVA analyses were significantly different.

If normality could not be assumed for the data included in the two-way ANOVA and/or the variances between the treatment groups being compared in the two-way ANOVA analyses were significantly different, the analyses mentioned above were performed separately depending on the comparison being made and the assumptions (normality of data and/or homogeneity of variance) violated.

# Chapter 3: Results

The results section is divided into parts A, B, and C, which are briefly outlined below.

Part (A) Aminoglycoside-induced cochlear toxicity: The results related to the gentamicin and amikacin-induced cochlear toxicity are presented separately followed by the results from analyses comparing the cochlear toxicity between the two aminoglycosides treatment groups.

Part (B) Aminoglycoside-induced vestibular toxicity: The results related to the gentamicin and amikacin-induced vestibular toxicity are presented separately followed by the results from analyses comparing the vestibular toxicity between the two aminoglycosides treatment groups.

Part (C) TGP treatment: The results from analyses comparing the aminoglycoside alone and the aminoglycoside + TGP treatment groups are presented.

# PART A: Aminoglycoside-induced cochlear toxicity

### 3.1) Gentamicin-induced cochlear toxicity

No IHC or OHC loss was observed in the control cochlear explants (Figure 6A). Approximately 40% IHC loss was observed in the cochlear explants treated with the lowest dose of gentamicin that is, gentamicin 0.3 mM (Figure 6A). Progressive IHC and OHC loss was observed in the cochlear explants treated with increased gentamicin concentration (Figure 6A). Near complete IHC and OHC loss was observed in the explants treated with the maximum dose of gentamicin, that is gentamicin 2.4 mM (Figure 6A). Compared to the OHC loss, a relatively greater IHC loss was observed in all the cochlear explants treated with gentamicin (Figure 6A).

The IHC survival data of the gentamicin-treated cochlear explant groups were normally distributed ($p = 0.598$). The variances in the IHC survival data between the gentamicin treatment groups were found to be approximately equal ($p = 0.205$). A linear regression analysis was used to evaluate the IHC data of the gentamicin treatment groups. The goodness of fit ($R^2$) for the linear regression was 0.811. The $TC_{50}$ for gentamicin-induced reduction in IHC survival was calculated to be approximately 0.4 mM. The mean IHC survival (% of control) rates ± SEM for the gentamicin 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated explants were 57.31 ± 10.20, 44.75 ± 5.61, 24.03 ± 6.30, 41.47 ± 5.17 and 3.33 ± 2.50, respectively ($n = 3$) (Figure 6B).

The OHC survival data for gentamicin-treated cochlear explants were found to be normally distributed ($p = 0.363$). The variances for the OHC survival rate data in the gentamicin treatment groups were found to be approximately equal ($p = 0.221$). A non-linear (sigmoidal) regression was used to analyze the OHC data of the gentamicin-treated cochlear explants. The goodness of fit ($R^2$) for the non-linear regression was 0.994 (Figure 6C). The $TC_{50}$ for gentamicin-induced OHC

survival reduction was calculated to be approximately 1.140 mM. The mean OHC survival rate (% of control) ± SEM for the gentamicin 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated explants were 72.63 ± 10.3, 77.78 ± 14.60, 72.46 ± 12.35, 60.63 ± 15.63 and 16.70 ± 0.86, respectively (n = 3) (Figure 6C).

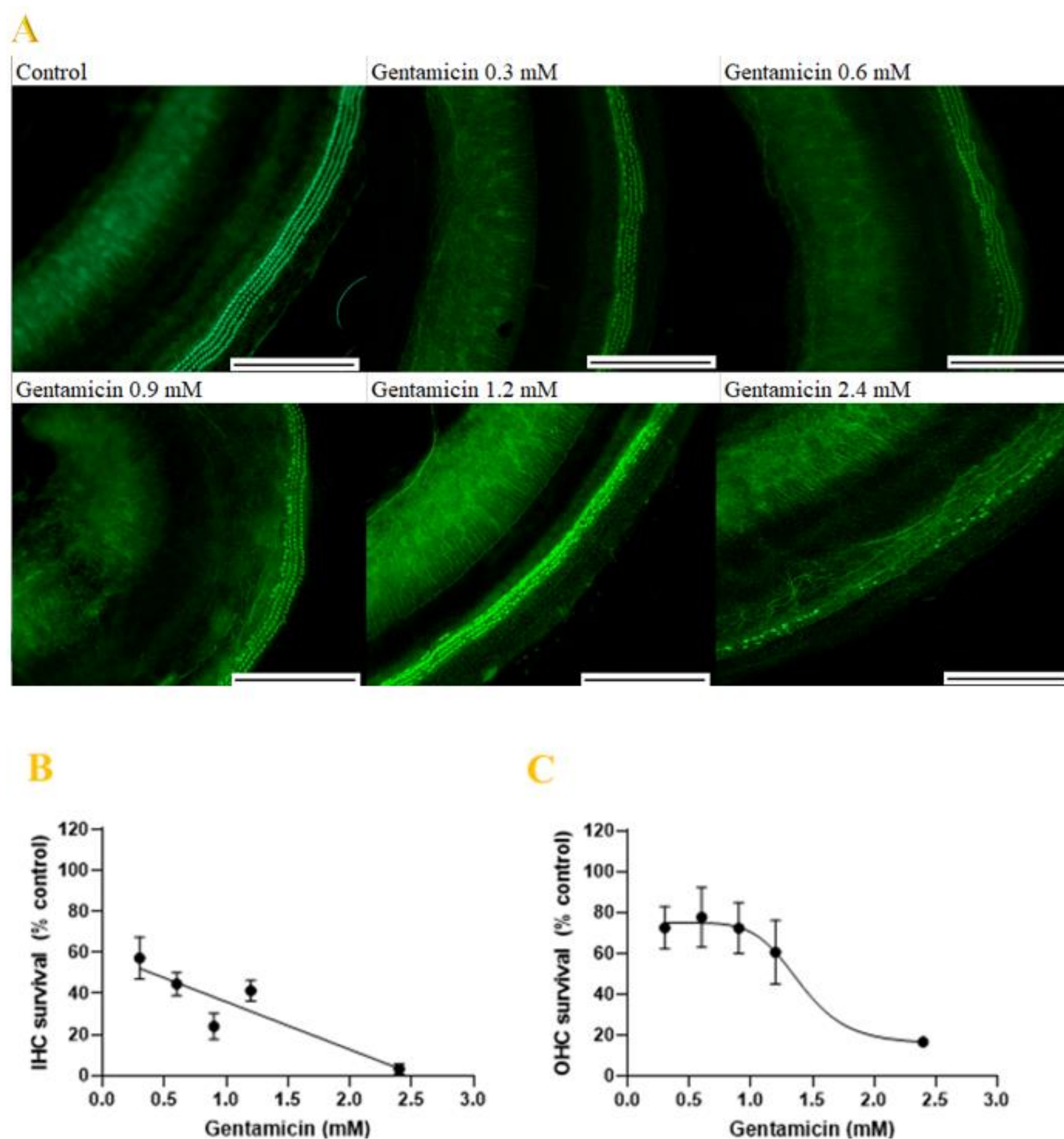



**Figure 6**. Representative pictures of the cochlear explants treated with different concentrations of gentamicin, stained with fluorophore-conjugated phalloidin, and observed under a fluorescent microscope (**A**). Scale bar = 200 μm. Effects of increasing gentamicin concentrations on IHC survival (% of control) (**B**) and OHC survival (% of control) (**C**). Data presented as mean ± SEM.

**3.2) Amikacin-induced cochlear toxicity**

No IHC or OHC loss was observed in the control cochlear explants (Figure 7A). Mild IHC loss was observed in the explants that were treated with amikacin 0.3 mM and 0.6 mM while moderate IHC loss was observed in the cochlear explants treated with amikacin 0.9 mM (Figure 7A). Meanwhile, extensive IHC damage was observed in the cochlear explants that were treated with amikacin 1.2 mM and amikacin 2.4 mM (Figure 7A). In contrast to the IHC loss, no OHC loss was observed in any explants treated with amikacin except for the explants treated with amikacin 2.4 mM where limited OHC loss was observed (Figure 7A).

The IHC survival rate data for amikacin-treated cochlear explants were found to be normally distributed ($p = 0.227$). The variances for the IHC survival rate data in the amikacin treatment groups were found to be approximately equal ($p = 0.286$). Non-linear (sigmoidal) regression was used to analyze the IHC data of the amikacin-treated cochlear explants and the goodness of fit ($R^2$) for the non-linear regression was 0.993 (Figure 7B). The $TC_{50}$ for amikacin-induced IHC survival reduction was approximately 0.903 mM. The mean IHC survival rate (% control) for the amikacin 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated explants were 87.73 ± 9.62 (n = 3), 82.61 ± 8.90 (n = 3), 62.85 ± 11.89 (n = 3), 34.98 ± 22.05 (n = 2) and 37.76 ± 5.97 (n = 3), respectively (Figure 7B).

Since the OHC survival rate data was not normally distributed ($p = 0.028$) and since the variances between the treatment groups included in the regression analyses were significantly different ($p = 0.02$), non-linear (sigmoidal) regression was not used to analyze the OHC data of the amikacin-treated cochlear explants. Nonetheless, only 20% OHC survival rate reduction (% control) was observed in the maximum amikacin concentration (2.4 mM) (Figure 7C) so, the $TC_{50}$ for amikacin-induced OHC survival reduction was estimated to be greater than the maximum dosage used in

this experiment, that is, > 2.4 mM. The mean OHC survival rate (% control) for the amikacin 150 μM, 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated explants were 96.66 ± 3.29, 98.45 ± 1.17, 99.49 ± 0.73, 97.22 ± 2.79 and 79.92 ± 10.17, respectively (n = 3) (Figure 7C).

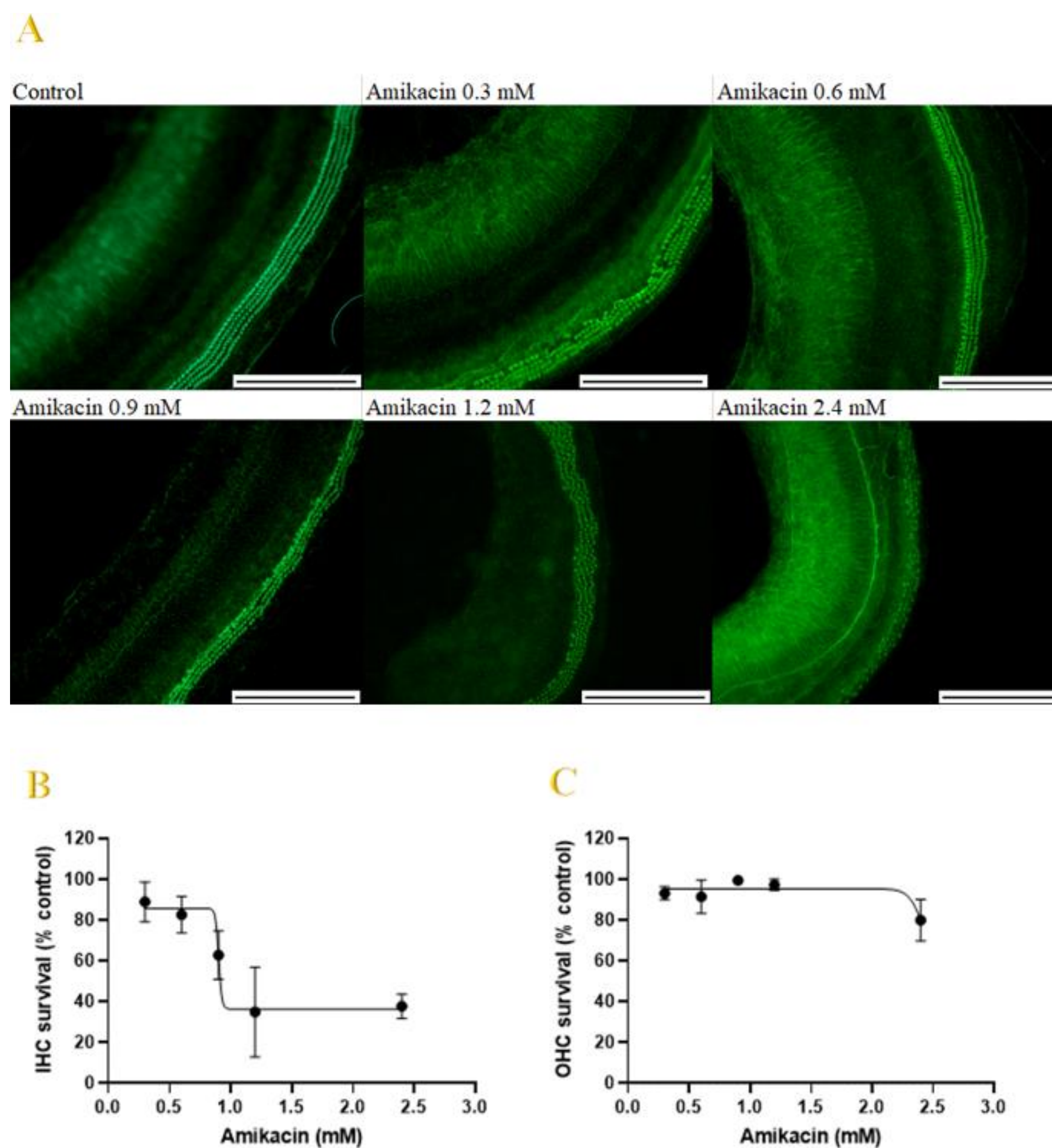



**Figure 7.** Representative pictures of the cochlear explants treated with different concentrations of amikacin, stained with fluorophore-conjugated phalloidin, and observed under a fluorescent microscope (**A**). Scale bar = 200 μm. Effects of increasing amikacin concentrations on IHC survival (% of control) (**B**) and OHC survival (% of control) (**C**). Data presented as mean ± SEM.

**3.3) Comparison between gentamicin and amikacin-induced cochlear toxicity**

a) IHC toxicity

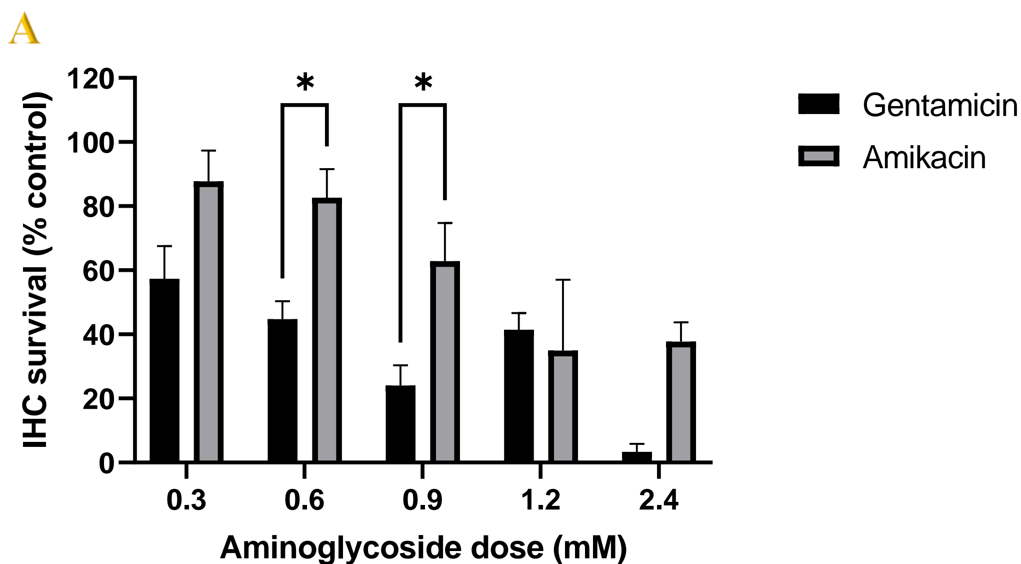
The data used in the two-way ANOVA that is, the IHC survival data from gentamicin and amikacin treatment groups, were normally distributed ($p = 0.655$). The variances for the IHC survival rate data in the gentamicin and amikacin treatment groups were found to be approximately equal ($p = 0.077$). The aminoglycoside type was found to have a significant effect on the IHC survival [($F_{(1.19)} = 22.95$, $p = 0.0001$]. Furthermore, using a Bonferroni post hoc analysis, significant differences in the IHC survival rate (% control) were observed between the gentamicin 0.6 mM versus amikacin 0.6 mM treatment groups ($p = 0.031$) and the gentamicin 0.9 mM versus amikacin 0.9 mM treatment groups ($p = 0.026$) (Fig. 8A).

The aminoglycoside dose was also found to have a significant effect on IHC survival [$F_{(4,19)} = 11.22$, $p < 0.0001$], and the Bonferroni post-hoc test revealed that there were significant differences between the gentamicin 0.3 mM versus gentamicin 2.4 mM treatment groups ($p = 0.0032$), the gentamicin 0.6 mM versus gentamicin 2.4 mM treatment groups ($p = 0.032$), the amikacin 0.3 mM versus amikacin 1.2 mM treatment groups ($p = 0.0112$), the amikacin 0.3 mM versus amikacin 2.4 mM treatment groups ($p = 0.0067$), the amikacin 0.6 mM versus amikacin 1.2 mM treatment groups ($p = 0.0261$) and the amikacin 0.6 mM versus amikacin 2.4 mM treatment groups ($p = 0.0172$) (Fig 8B).

Furthermore, separate one-way analyses were used to confirm that both gentamicin and amikacin doses had a significant effect on IHC survival rate (% control) [$F_{(4,10)} = 10.53$, $p = 0.0013$] and [$F_{(4,9)} = 4.750$, $p = 0.0245$], respectively.

The same data were used for the one-way ANOVAs and the regressions analyses. Similar to the regression analysis, normality and the homogeneity of variance could be assumed for all the one-way ANOVA analyses except for the one-way ANOVA using OHC survival rate data from the amikacin-treated cochlear explants.

A




B

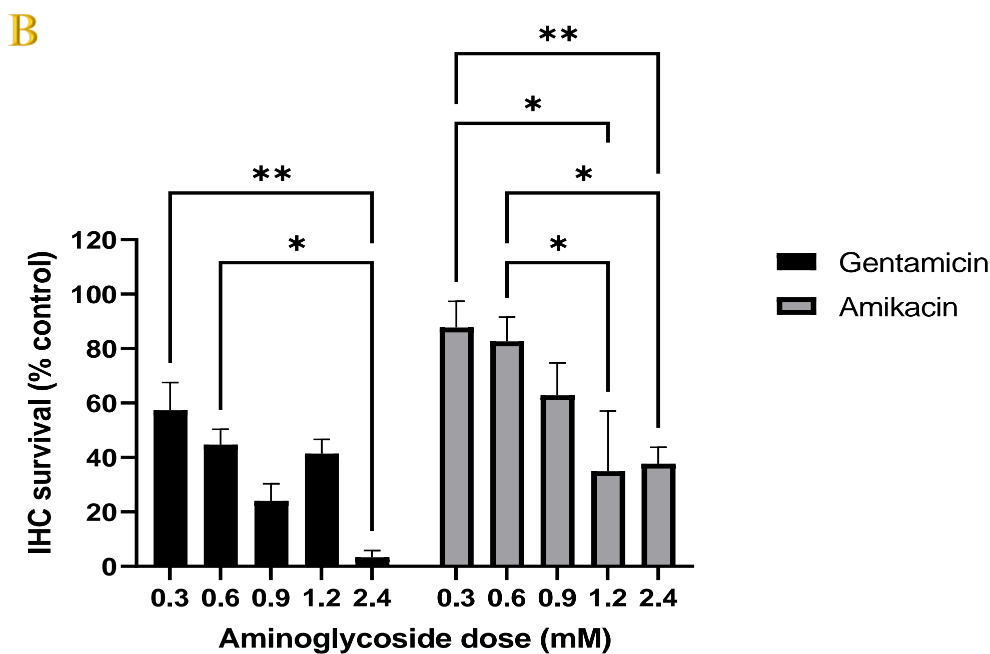



**Figure 8.** The effect of the aminoglycoside type on the IHC survival is shown by comparing the effects of the corresponding gentamicin and amikacin doses on the IHC survival rate (% control) (**A**). The effect of the aminoglycoside dose is shown by comparing the effects of the different gentamicin and amikacin doses on IHC survival rate (% control) (**B**).

b) OHC toxicity

The variances for the OHC survival data in the gentamicin and amikacin treatment groups were significantly different ($p = 0.012$) and the data not normally distributed ($p = 0.0494$). Hence, instead of using a two-way ANOVA, separate analyses (that is, either the Mann Whitney, Welch, or unpaired t-test) were used to compare the effects of the corresponding gentamicin and amikacin doses on the OHC survival rates (% control) to evaluate the effects of the aminoglycoside type.

The OHC survival data of both the gentamicin 0.3 mM and amikacin 0.3 mM treatment groups were found to be normally distributed ($p = 0.876$ and 0.789, respectively) and the variances between the two groups were approximately equal ($p = 0.081$). Hence, an unpaired t-test was used and it was found that the OHC survival rates (% control) between the gentamicin 0.3 mM and amikacin 0.3 mM treatment groups were not significantly different (Figure 9A).

The OHC survival data in both gentamicin 0.6 mM and the amikacin 0.6 mM treatment groups were found to be normally distributed ($p = 0.963$ and 0.811, respectively) and the variances between the two groups were approximately equal ($p = 0.064$). Hence, an unpaired t-test was used and it was found that the OHC survival rates (% control) between the gentamicin 0.6 mM and amikacin 0.6 mM treatment groups were not significantly different ($p = 0.231$) (Figure 9A).

The normality of the OHC survival data could not be assumed for the amikacin 0.9 mM treatment group so, the Mann-Whitney test was used and it was found that the OHC survival rates (% control) between the gentamicin 0.9 mM and amikacin 0.9 mM treatment groups were not significantly different ($p = 0.100$) (Figure 9A).

The OHC survival data for both the gentamicin 1.2 mM and the amikacin 1.2 mM treatment groups were found to be normally distributed ($p = 0.100$ and 0.787, respectively) and the variances

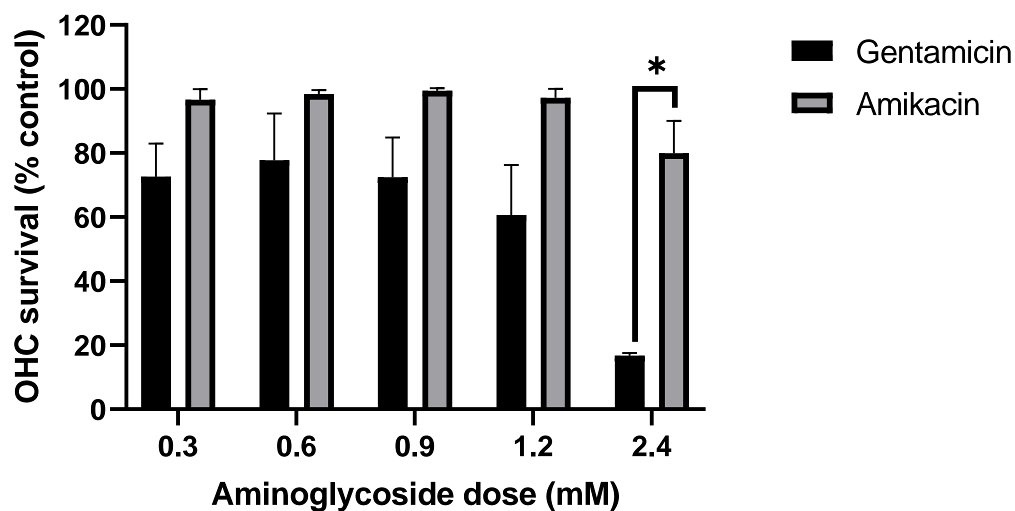
between the two groups were approximately equal ($p = 0.178$). Hence, an unpaired t-test was used and it was found that the OHC survival rates (% control) between the gentamicin 1.2 mM and the amikacin 1.2M treatment groups were not significantly different ($p = 0.083$) (Figure 9A).

The OHC survival data for both the gentamicin 2.4 mM and the amikacin 2.4 mM treatment groups were found to be normally distributed ($p > 0.999$ and $p = 0.132$, respectively). However, the variances between the two treatment groups were significantly different ($p = 0.023$). Hence, the Welch test was used and it was found that there was a significant difference in the OHC survival rate (% control) between the gentamicin 2.4 mM and the amikacin 2.4 mM treatment groups ($p = 0.024$) (Figure 9A).

In order to evaluate the aminoglycoside dose effect on OHC survival, the OHC survival data (% control) from the gentamicin and the amikacin treatment groups were analyzed separately. The variances between the amikacin treatment groups were significantly different ($p = 0.002$) and the OHC survival rate (% control) data for the amikacin-treated cochlear explants were not normally distributed ($p = 0.0279$). Hence, a non-parametric Kruskal-Wallis test was used and it was found that the amikacin doses did not have a significant effect on OHC survival ($p = 0.645$) (Figure 9A).

The OHC survival data for the gentamicin treatment groups were normally distributed ($p = 0.363$) and the variances between the gentamicin treatment groups were approximately equal ($p = 0.221$). Hence, a one-way ANOVA was used and it was found that the gentamicin doses had a significant effect on the OHC survival rates (% control) [$F_{(4,10)} = 4.73$, $p = 0.0267$]. However, upon using a Bonferroni post-hoc test, no significant differences were observed in OHC survival rates between the gentamicin treatment groups (Figure 9B).

A

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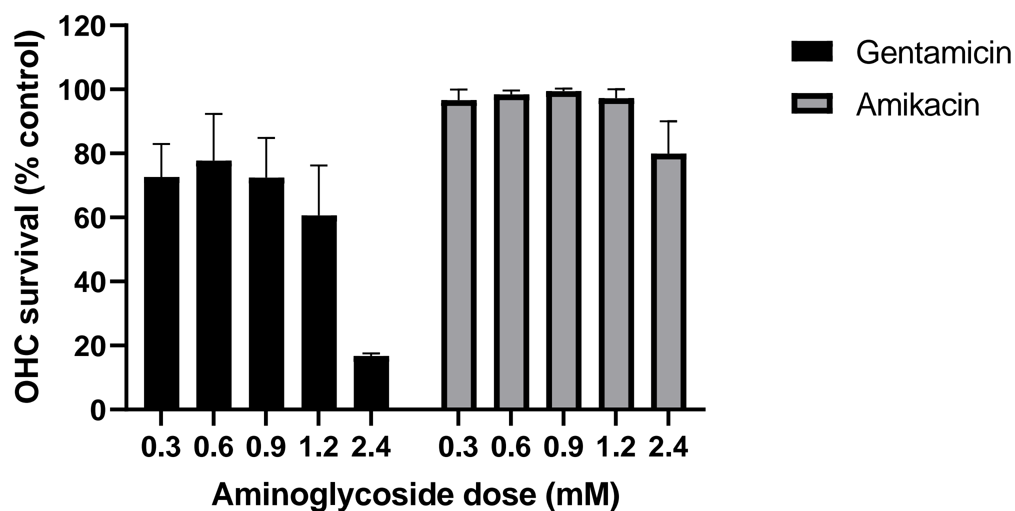



**Figure 9.** The effect of the aminoglycoside type on OHC survival is shown by comparing the effects of the corresponding gentamicin and amikacin doses on the OHC survival rate (% control) (**A**). The effect of the aminoglycoside doses on OHC survival is shown by comparing the effects of the different gentamicin and amikacin doses on the OHC survival rate (% control) (**B**).

# PART B: Aminoglycoside-induced vestibular toxicity

### 3.4) Gentamicin-induced vestibular toxicity

Utricular HC loss was not observed in the control utricular explants (Fig. 10A). Gradual utricular HC loss was observed with increasing gentamicin concentration (Fig. 10A).

The residuals of the utricular HC survival data for the gentamicin-treated utricular explants were not normally distributed ($p = 0.0325$). However, the utricular HC survival (% control) data were found to be normally distributed ($p = 0.890$) and the variances of the utricular HC survival data were found to be approximately equal ($p = 0.528$). Hence, a linear regression was used to analyze the utricular HC survival (% control) data of the gentamicin treatment groups and the goodness of fit ($R^2$) for the linear regression was 0.997 (Figure 10B). The $TC_{50}$ for gentamicin-induced reduction of the utricular HC survival was calculated to be approximately 2.096 mM. The mean utricular HC survival rate (% of control) ± SEM for the gentamicin 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated utricular explants were 83.54 ± 5.56, 75.80 ± 8.45, 72.29 ± 7.53, 66.26 ± 5.23 and 42.99 ± 4.77, respectively ($n = 3$).

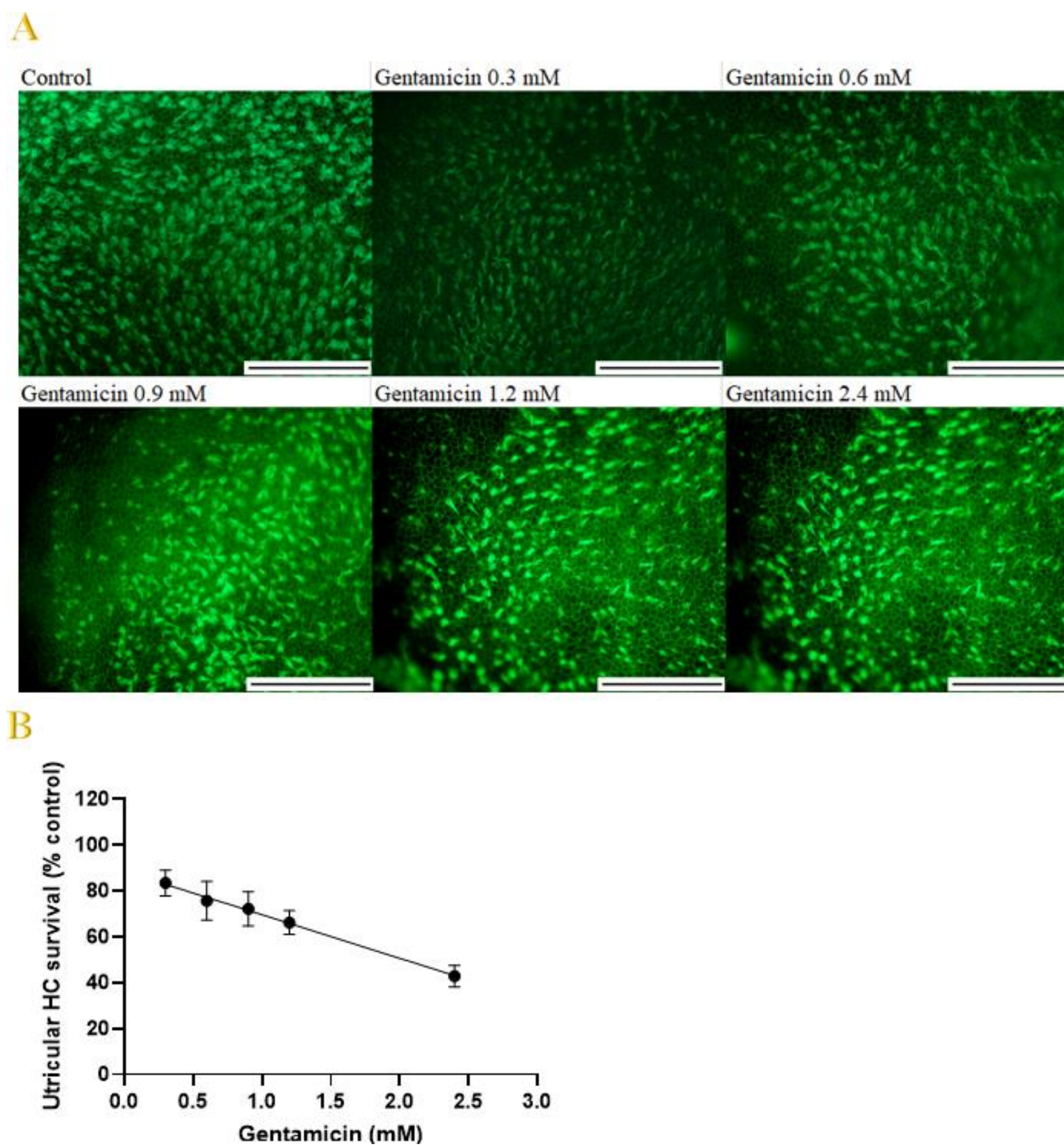



**Figure 10**. Representative pictures of the utricular explants treated with different concentrations of gentamicin, stained with fluorophore-conjugated phalloidin, and observed under a fluorescent microscope (**A**). Scale bar = 100 µm. Effects of increasing gentamicin concentrations on utricular HC survival (% control) (**B**). Data presented as mean ± SEM.

**3.5) Amikacin-induced vestibular toxicity**

Utricular HC loss was not observed in the explants treated with the different doses of amikacin (Figure 11A). The utricular HC survival rate data for the amikacin-treated utricular explants were found to be normally distributed ($p = 0.693$) and the variances of the utricular HC survival data between the amikacin treatment groups were found to be approximately equal ($p = 0.210$). Hence, a non-linear regression was used to analyze the utricular HC survival of the amikacin-treated utricular explants and the goodness of fit ($R^2$) for the non-linear regression was 0.768 (Figure 11B). The use of maximum amikacin concentration (2.4 mM) in our study resulted in only a 12% utricular HC survival reduction (% control) (Figure 11B). Hence, the $TC_{50}$ for amikacin-induced utricular HC survival reduction was estimated to be greater than the maximum amikacin concentration used in this experiment, that is, > 2.4 mM. The utricular HC survival (% of control) for the amikacin 0.3 mM, 0.6 mM, 0.9 mM, 1.2 mM and 2.4 mM treated explants were 93.31 ± 2.61(n = 3), 98.07 ± 5.00 (n = 4), 95.29 ± 4.73(n = 4), 97.20 ± 2.31 (n = 3) and 88.50 ± 3.20 (n = 3), respectively (Figure 11B).

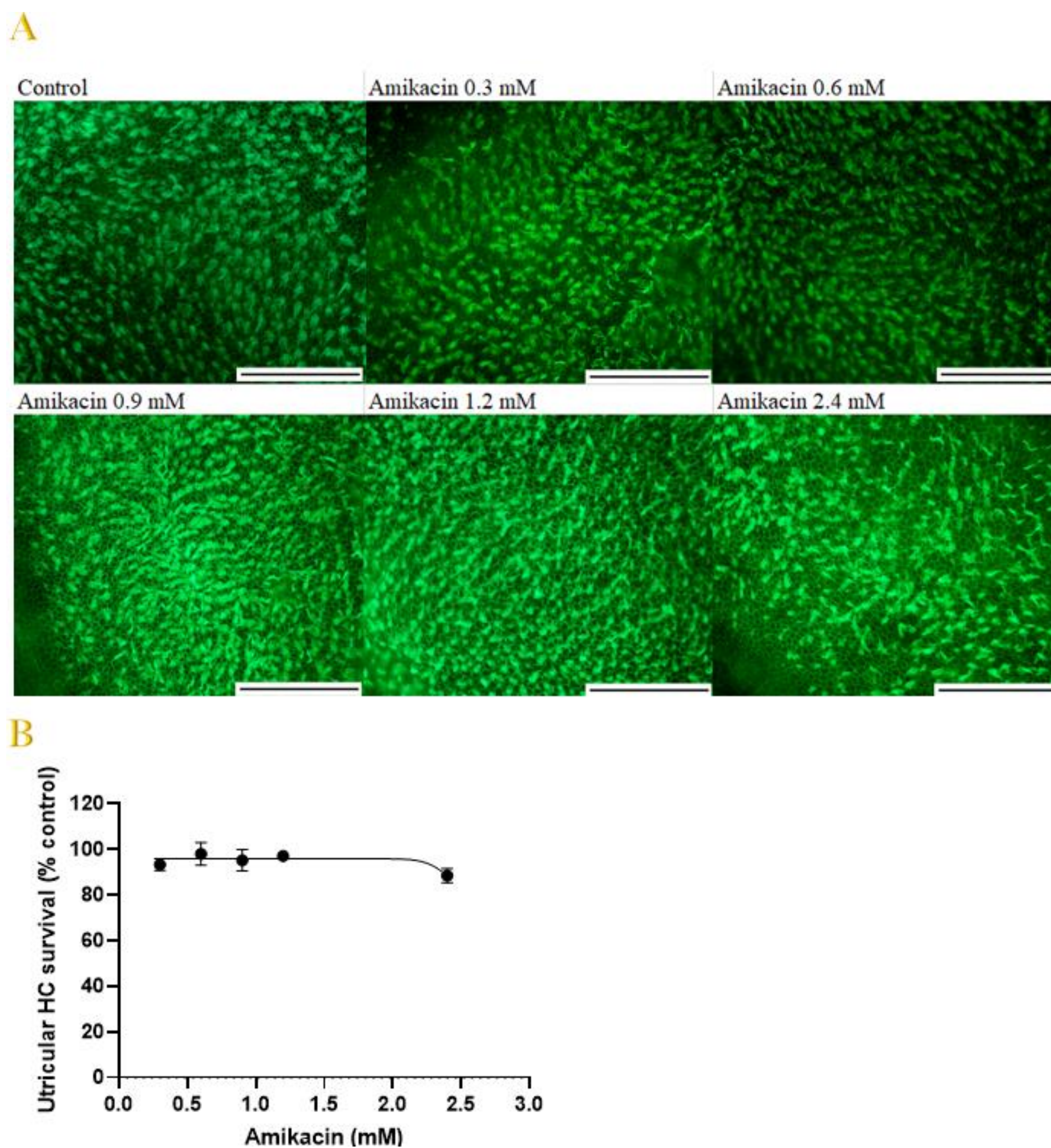



**Figure 11.** Representative pictures of the utricular explants treated with different concentrations of amikacin, stained with fluorophore-conjugated phalloidin, and observed under a fluorescent microscope (**A**). Scale bar = 100µm. Effects of increasing amikacin concentrations on OHC survival rate (% of control) (**B**). Data presented as mean ± SEM.

**3.6) Comparison between gentamicin and amikacin-induced vestibular toxicity**

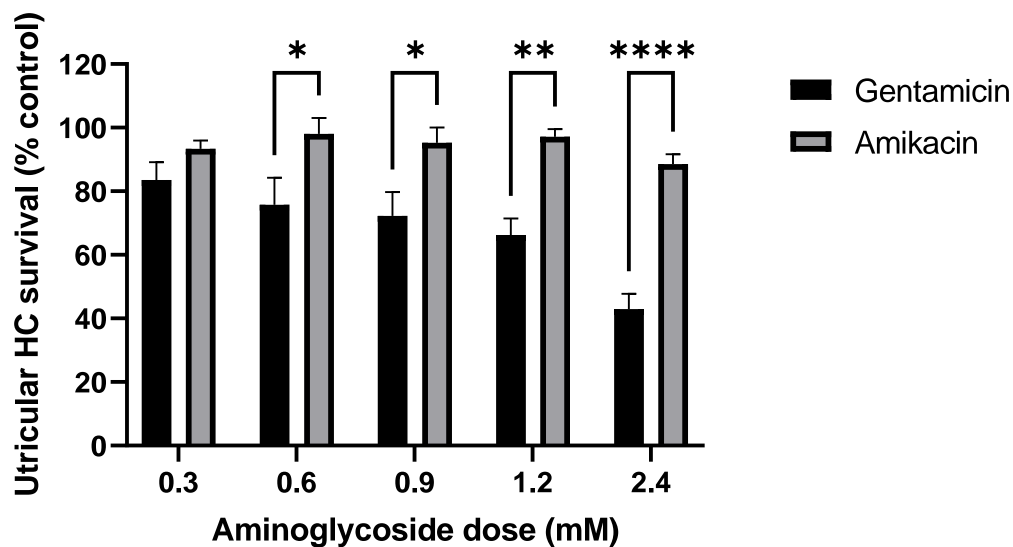
The data used in the two-way ANOVA that is, the utricular HC survival data from gentamicin and amikacin treatment groups, were normally distributed ($p = 0.194$). The variances in the utricular HC survival rate data between the groups being compared, that is, the gentamicin and amikacin treatment groups were found to be approximately equal ($p = 0.203$).

The aminoglycoside type had a significant effect on the utricular HC survival (% control) ($F_{(1,22)} = 61.47$, $p < 0.0001$). Furthermore, a significant difference in the utricular HC survival rate (% control) was observed between the gentamicin 0.6 mM versus amikacin 0.6 mM treatment groups ($p = 0.0265$), the gentamicin 0.9 mM versus amikacin 0.9 mM treatment groups ($p = 0.0208$), the gentamicin 1.2 mM versus amikacin 1.2 mM treatment groups ($p = 0.0029$), and the gentamicin 2.4 mM versus amikacin 2.4 mM treatment groups ($p < 0.0001$) (Figure 12A).

The aminoglycosides doses also had a significant effect on utricular HC survival ($F_{(4,22)} = 5.67$, $p = 0.0027$). Using a Bonferroni post-hoc analysis, a significant difference in the utricular HC survival (% control) was observed between the gentamicin 0.3 mM versus gentamicin 2.4 mM treatment groups ($p = 0.0003$), the gentamicin 0.6 mM versus gentamicin 2.4 mM treatment groups ($p = 0.003$), and the gentamicin 0.9 mM versus gentamicin 2.4 mM treatment groups ($p = 0.010$). The one-way ANOVAs were used to find that only the gentamicin doses had a significant effect on the utricular HC survival [$F_{(4,10)} = 5.676$, $p = 0.0120$] while the amikacin doses did not have a significant effect on the utricular HC survival [$F_{(4,22)} = 2.93$, $p = 0.0439$] (Figure 12B).

A significant interaction effect was observed between the effects of aminoglycoside type and aminoglycoside dose on utricular HC survival [$F_{(4,22)} = 2.93$, $p = 0.0439$].

A




B

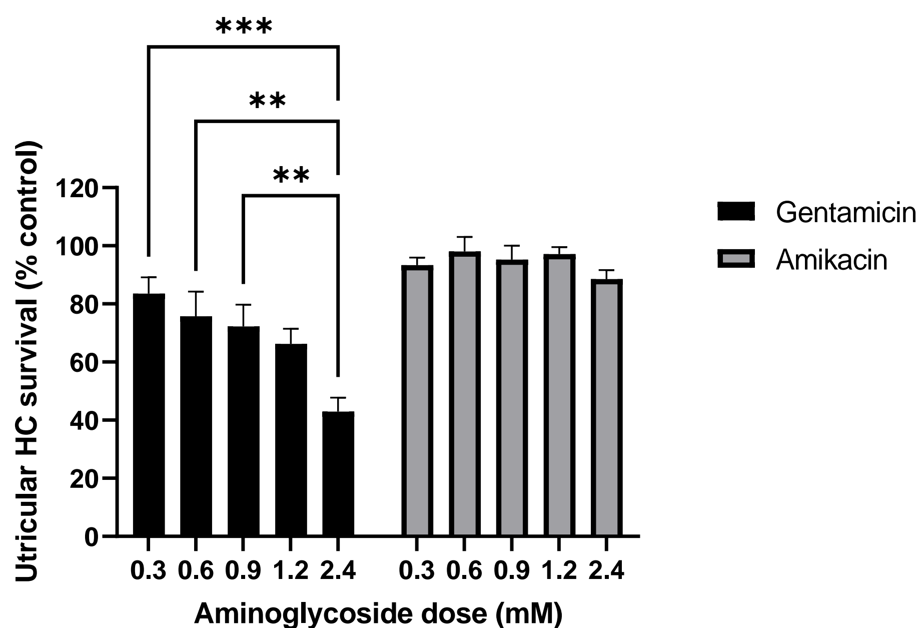



**Figure 12.** The effect of aminoglcyoside type on utricular HC survival illustrated after comparing the effects of the corresponding gentamicin and amikacin doses on utricular HC survival rate (% control) **(A)**. The effect of aminoglcyoside dose on OHC survival illustrated by comparing the effects of the different getamicin and amkiacin doses on OHC survival rate (% control) **(B)**.

| Condition | $TC_{50}$ value |
|---|---|
| Gentamicin-induced reduction in IHC survival | 0.4 mM |
| Amikacin-induced reduction in IHC survival | 0.903 mM |
| Gentamicin-induced reduction in OHC survival | 1.140 mM |
| Amikacin-induced reduction in OHC survival | > 2.4 mM |
| Gentamicin-induced reduction of the utricular HC survival | 2.096 mM |
| Amikacin-induced reduction of the utricular HC survival | >2.4 mM |

**Table 3**. Summary of the $TC_{50}$ values obtained in relation to aminoglcysoide-induced HC toxicity

# PART C: TGP Treatment.

### 3.7) TGP treatment did not attenuate aminoglycoside-induced cochlear toxicity.

We did not observe a substantial difference in the IHC and OHC survival between the gentamicin 0.3 mM alone (Fig. 13A) and gentamicin 0.3 mM + TGP 100µM treatment groups (Fig. 13B). Normality could not be assumed for the IHC data of the gentamicin 0.3 mM + 100µM treatment group. Hence, the non-parametric Mann Whitney test was used and it was found that there was no significant difference in the IHC survival rate between the gentamicin 0.3 mM alone (n = 3) and gentamicin 0.3 mM + TGP 100µM (n = 2) treatment groups (p = 0.800) (Figure 13C).

The OHC survival data of both the gentamicin 0.3 mM alone and gentamicin 0.3 mM + 100µM treatment groups were found to be normally distributed (p = 0.312 and 0.160, respectively). However, the difference in the variance of O HC survival (% control) data between the gentamicin 0.3 mM + TGP 100µg/ml and gentamicin 0.3 mM groups was significant (p = 0.038) so, the Welch test was used and it was found that there was no significant difference in the OHC survival rate between the gentamicin 0.3 mM alone (n = 3) and gentamicin 0.3 mM + TGP 100µM (n = 3) treatment groups (p = 0.124) (Figure 13D).

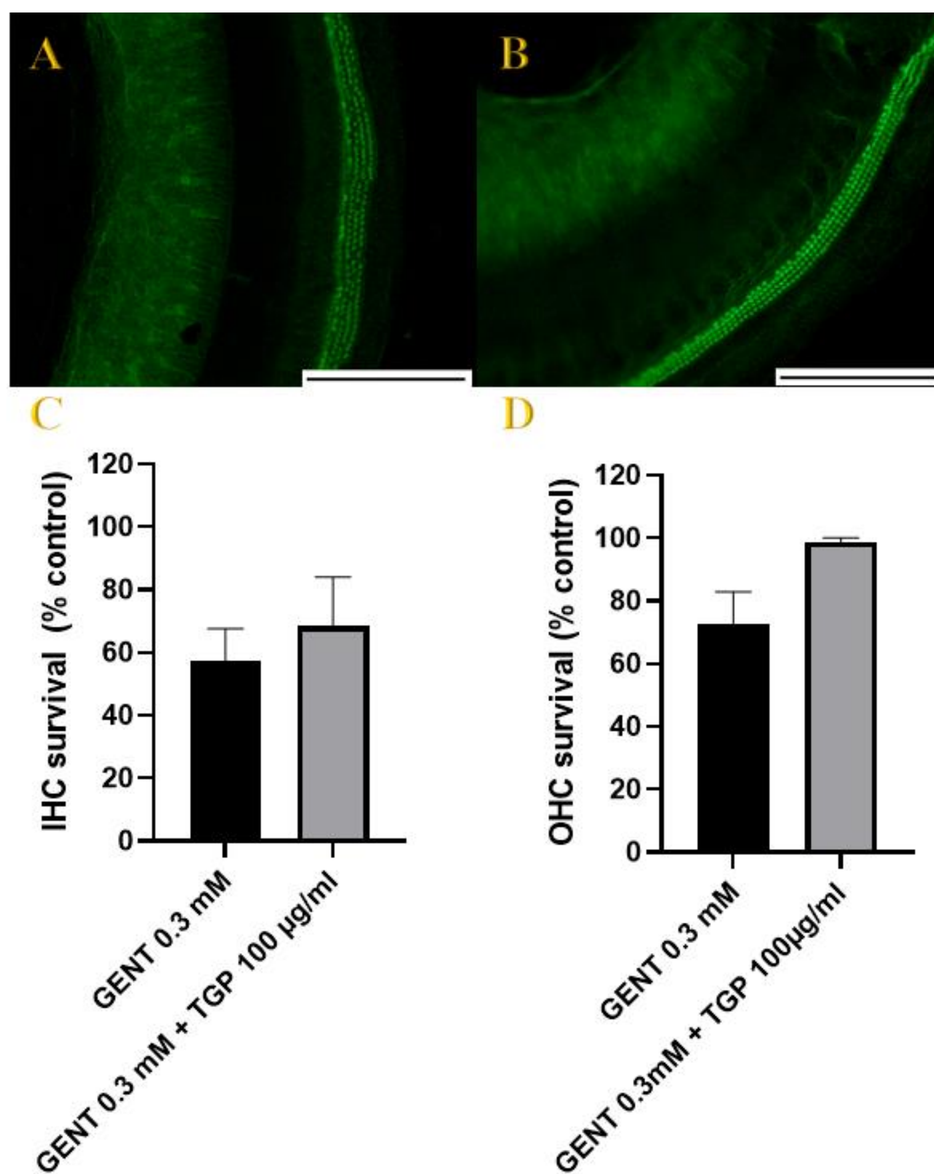



**Figure 13.** Representative pictures of the cochlear explants treated with gentamicin 0.3 mM alone (**A**) and gentamicin 0.3 mM + TGP 100 µg/ml (**B**), stained with fluorophore-conjugated phalloidin and observed under a fluorescent microscope. Scale bar = 200 µM. Comparison between the effects of gentamicin 0.3 mM alone and gentamicin 0.3 mM + TGP 100 µg/ml treatment on IHC survival rate (% control) (**C**) and OHC survival rate (% control) (**D**). Data presented as mean ± SEM. GENT = Gentamicin.

We did not observe a substantial difference in the IHC and OHC survival between the gentamicin 0.9 mM alone (Figure 14A) and gentamicin 0.9 mM + TGP 100µM (Figure 14B) treated cochlear explants. The IHC survival data of both the gentamicin 0.9 mM alone and gentamicin 0.9 mM + 100µM treatment groups, were found to be normally distributed ($p = 0.069$ and 0.411, respectively). The variances of the IHC survival data between the gentamicin 0.9 mM + TGP 100µg/ml and gentamicin 0.9 mM treatment groups were approximately equal ($p = 0.718$). Thus, an unpaired t-test was used and it was found that the difference in the IHC survival rate between gentamicin 0.9 mM alone ($n = 3$) and gentamicin 0.9 mM + TGP 100µM ($n = 3$) treatment groups was not significant ($p = 0.067$) (Figure 14C).

The OHC survival data of both gentamicin 0.9 mM alone and gentamicin 0.9 mM + 100µM treatment groups, were found to be normally distributed ($p = 0.0807$ and 0.0949, respectively). However, the difference in variance of OHC survival (% control) data between the gentamicin 0.9 mM + TGP 100 µg/ml and gentamicin 0.9 mM was significant ($p = 0.015$). Hence, the Welch test was used and it was found the difference in the OHC survival rate between the gentamicin 0.9 mM alone ($n = 3$) and gentamicin 0.9 mM + TGP 100µM ($n = 3$) treatment groups was not significant ($p = 0.188$) (Figure 14D).

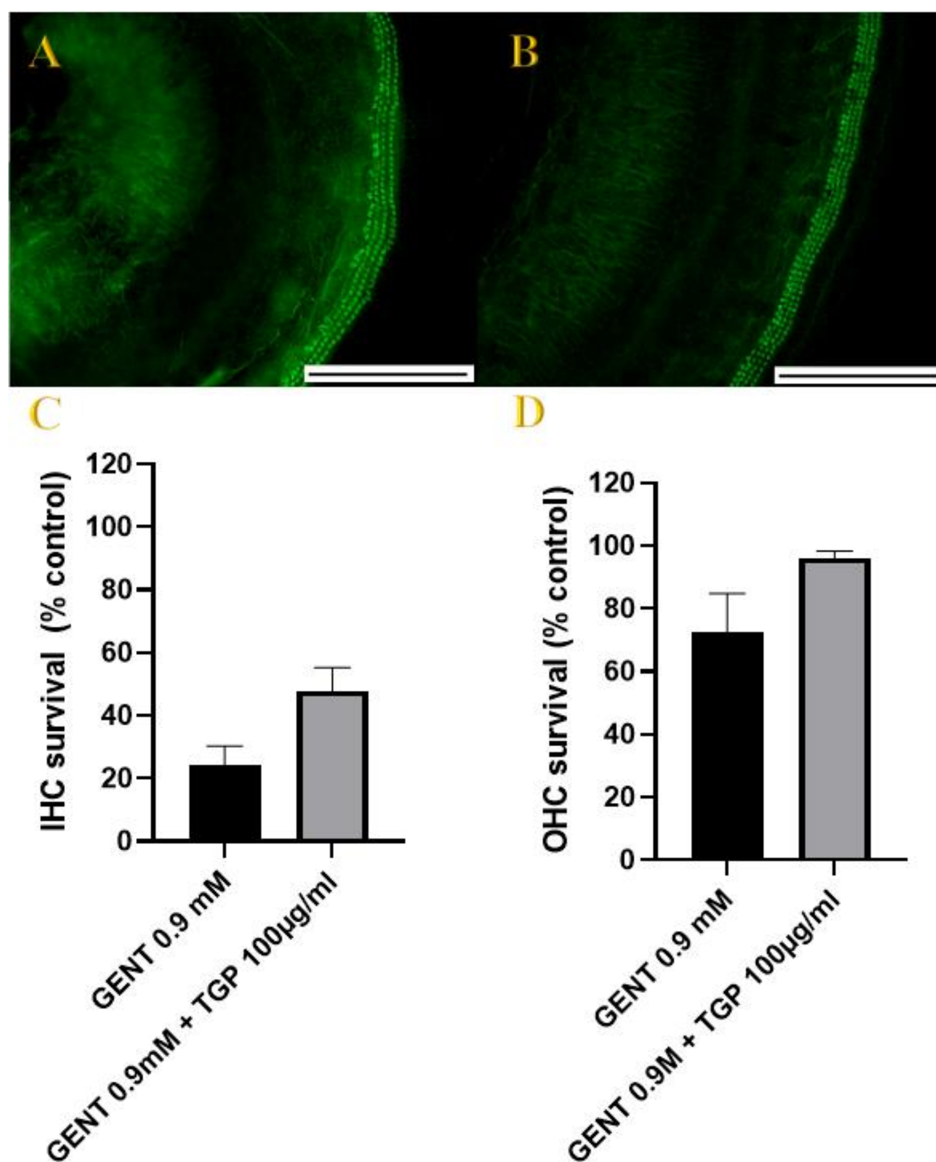



**Figure 14.** Representative pictures of the cochlear explants treated with gentamicin 0.9 mM alone (**A**) and gentamicin 0.9 mM + TGP 100 µg/ml (**B**), stained with fluorophore-conjugated phalloidin and observed under a fluorescent microscope. Scale bar = 200 µm. Comparison between the effects of gentamicin 0.9 mM alone and gentamicin 0.9 mM + TGP 100 µg/ml treatment on IHC survival rate (% control) (**C**) and OHC survival rate (% control) (**D**). Data presented as mean ± SEM. GENT = Gentamicin.

We did not observe a considerable difference in the IHC and OHC survival rate between the amikacin 0.6 mM alone (Fig.15 A) and amikacin 0.6 mM + TGP 100µg/ml (Fig. 15 B) treated cochlear explants. The IHC survival data of both the amikacin 0.6 mM alone and amikacin 0.6 mM + 100µM treatment groups were found to be normally distributed ($p = 0.845$ and 0.988, respectively). The variances of IHC survival data between the amikacin 0.6 mM and amikacin 0.6 mM + TGP 100µg/ml treatment groups were not significantly different ($p = 0.169$). Hence, an unpaired t-test was used and it was found that the difference in the IHC survival rate between the amikacin 0.6 mM alone and amikacin 0.6 mM + TGP 100µM treatment groups was not significant ($p = 0.129$) (Figure 15C).

Normality could not be assumed for the OHC data in the amikacin 0.6 mM alone and amikacin 0.6 mM + 100µM treatment groups. Hence, the non-parametric Mann-Whitney test was used and it was found that the difference in the OHC survival rate between the amikacin 0.6 mM alone ($n = 3$) and amikacin 0.6 mM + TGP 100µM ($n = 3$) treatment groups was not significant ($p = 0.300$) (Figure 15D).

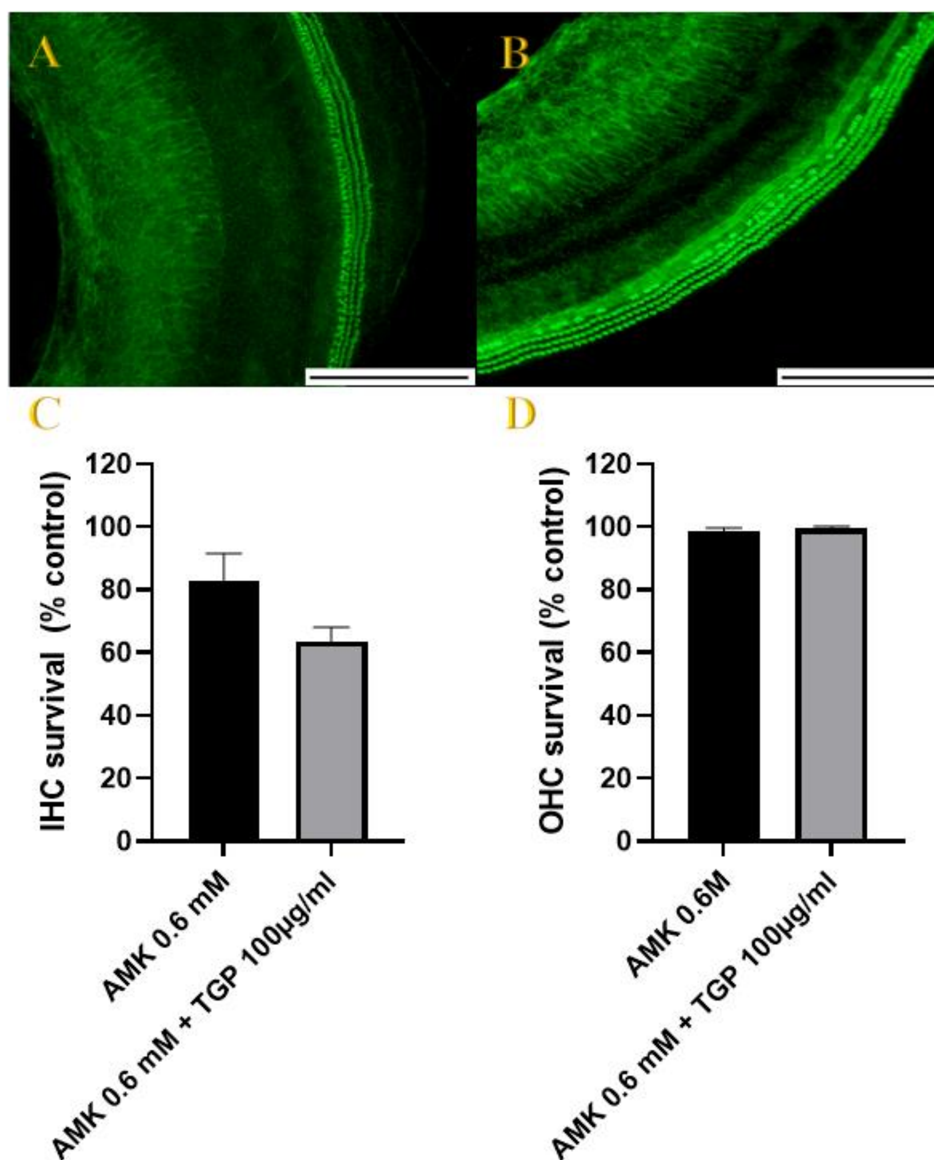



**Figure 15.** Representative pictures of the cochlear explants treated with amikacin 0.6 mM alone (**A**) and amikacin 0.6 mM + TGP 100 µg/ml (**B**), stained with fluorophore-conjugated phalloidin and observed under a fluorescent microscope. Scale bar = 200 µm. Comparison between the effects of amikacin 0.6 mM alone and amikacin 0.6 mM + TGP 100 µg/ml treatment on IHC survival rate (% control) (**C**) and OHC survival rate (% control) (**D**). Data presented as mean ± SEM. AMK = Amikacin.

### 3.8) TGP treatment attenuated aminoglycoside-induced vestibular toxicity

We observed a substantial increase in the utricular HC survival in the gentamicin 2.4 mM + TGP 100µg/ml treated utricular explants (Figure 16A) when compared to the gentamicin 2.4 mM alone treated utricular explants (Figure 16B).

The utricular HC survival data (% of control) of both gentamicin 2.4 mM alone and gentamicin 2.4 mM treatment groups, were found to be normally distributed (p-value = 0.661 and 0.679, respectively). The variances in the utricular HC survival data between the gentamicin 2.4 mM + TGP 100µg/ml and gentamicin 2.4 mM alone treatment groups were approximately equal (p-value = 0.668). Hence, an unpaired t-test was used and it was found that there was a significant increase in the utricular HC survival (by approximately 57%) in the gentamicin 0.9 mM + TGP 100µM (n = 3) treatment group when compared to the utricular HC survival in the gentamicin 0.9 mM alone (n = 3) treatment group (p = 0.037) (Figure 16C).

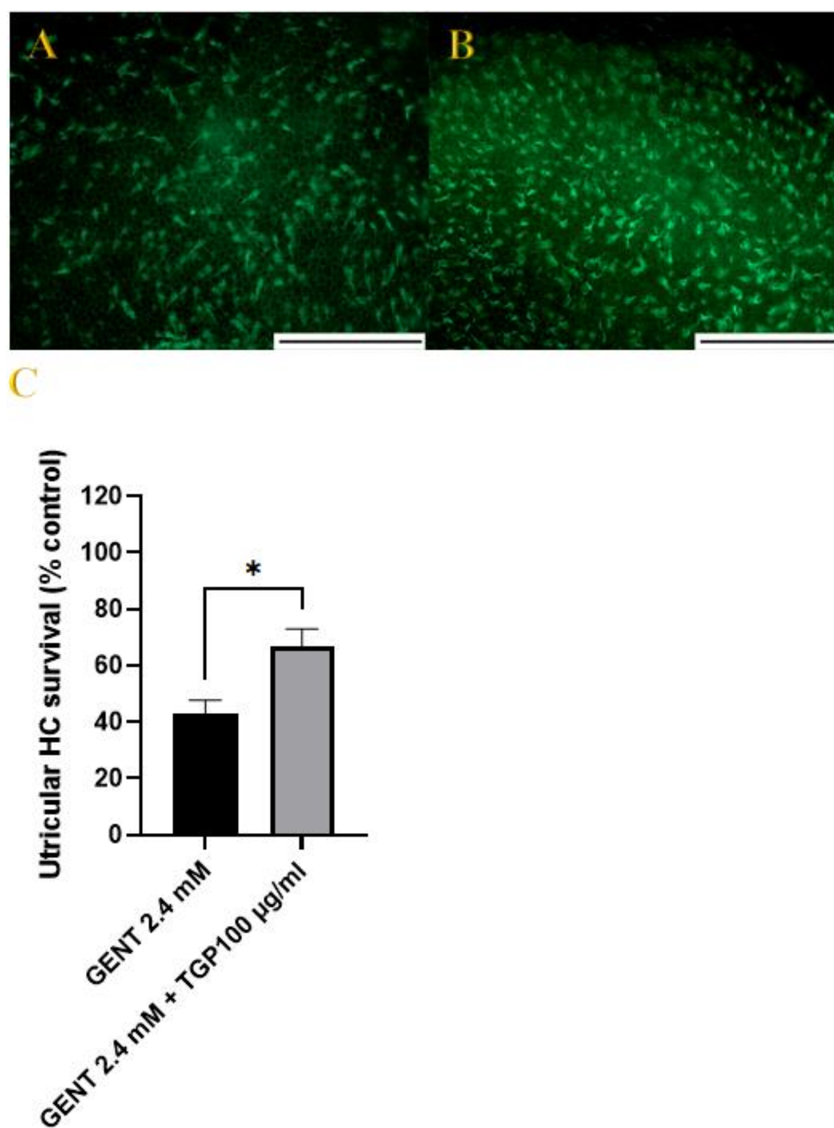



**Figure 16.** Representative pictures of the utricular explants treated with gentamicin 2.4 mM alone (**A**) and gentamicin 2.4 mM + TGP 100 µg/ml (**B**), stained with fluorophore-conjugated phalloidin and observed under a fluorescent microscope. Scale bar = 100 µM. Comparison between the effects of gentamicin 2.4 mM alone and gentamicin 2.4 mM + TGP 100µM treatment on utricular HC survival (% control) (**C**). Data presented as mean ± SEM. GENT = Gentamicin.

| Conditions compared | Treatment groups compared | Significant? | P – value |
| --- | --- | --- | --- |
| IHC survival | Gentamicin 0.3 mM alone VERSUS Gentamicin 0.3 mM + TGP 100µM | No | 0.800 |
| IHC survival | Gentamicin 0.9 mM alone VERSUS Gentamicin 0.9 mM + TGP 100µM | No | 0.067 |
| IHC survival | Amikacin 0.6 mM alone VERSUS Amikacin 0.6 mM + TGP 100µM | No | 0.129 |
| OHC survival | Gentamicin 0.3 mM alone VERSUS Gentamicin 0.3 mM + TGP 100µM | No | 0.124 |
| OHC survival | Gentamicin 0.9 mM alone VERSUS Gentamicin 0.9 mM + TGP 100µM | No | 0.188 |

| OHC survival | Amikacin 0.6 mM alone VERSUS Amikacin 0.6 mM + TGP 100µM | No | 0.300 |
|---|---|---|---|
| Utricular HC survival | Gentamicin 2.4 mM alone VERSUS Gentamicin 2.4 mM + TGP 100µM | Yes | 0.037 |

**Table 4**. Summary of the results obtained in relation to the TGP treatment.

# Chapter 4: Discussion

## 4.1) Gentamicin-induced ototoxicity

Generally, we observed a lower level of aminoglycoside-induced ototoxicity when compared to other *in-vitro* studies. For ease of comparison, the HC survival data from all the regions of the cochlea are averaged and presented as the overall cochlear HC survival from the studies where the HC survival was evaluated separately in the apex, middle, and base regions of the cochlear explant. Additionally, the HC loss in our study was calculated as the percent of HC missing when compared to the control explants. Therefore, the HC loss reported from this *in-vitro* study represents the percent of HC missing from the control explants in each study unless specified otherwise.

Nakamagoe et al. (2010) observed nearly 45% OHC loss after gentamicin (0.1 mM) treatment, in the cochlear explants of Sprague-Dawley rats, postnatal day 3-5 (P 3-5) (n = 13). Similarly, Mazurek et al. (2012) observed approximately 49% OHC loss following gentamicin (0.1 mM) treatment, in the cochlear explants of Wistar rats (P 3-5) (n = 10). Compared to these studies, we observed a similar level of OHC toxicity (approximately 44% OHC loss) after using a three-fold greater gentamicin dose (0.3 mM), in the cochlear explants of Wistar rats (P 2-4).

Additionally, Mazurek et al. (2012) found that gentamicin (0.1 mM) treatment could result in 43% IHC loss while we observed a similar level of IHC toxicity (approximately 43% IHC loss) after using a three-fold greater gentamicin dose (0.3 mM). Therefore, compared to previous *in-vitro* studies we observed a relatively lower gentamicin-induced OHC and IHC toxicity as we used higher gentamicin doses to achieve a similar level of IHC and OHC toxicity (Mazurek et al., 2012; Nakamagoe et al., 2010).

Lee et al. (2013) found that the cochlear explants of Sprague-Dawley rats (P 3-4), treated with gentamicin (0.3 mM) for 24 hours, 36 hours, and 48 hours exhibited approximately 15%, 57%, and 93% OHC loss, respectively (n = 3). Hence the results from Lee et al. (2013)'s study demonstrate that the duration of aminoglycoside exposure can drastically increase the aminoglycoside ototoxicity. The cochlear explants in both the Mazurek et al., (2012) and Nakamagoe et al. (2010)'s studies were exposed to gentamicin for 48 hours while the cochlear explants in our study were exposed to gentamicin for only 24 hours. Therefore, a longer duration of aminoglycoside exposure might be one of the reasons why Mazurek et al. (2012) and Nakamagoe et al. (2010) observed a greater level of gentamicin-induced IHC/OHC loss compared to our study.

Besides the duration of aminoglycoside exposure, the interval between the aminoglycoside exposure and HC loss evaluation might also affect the HC survival. Bramhall et al. (2014) found that gentamicin (0.05 mM) resulted in approximately 44% OHC loss in the cochlear explants of genetically modified mice. They did not specify the sample size. Nonetheless, compared to our study and the studies discussed previously (Mazurek et al., 2012; Nakamagoe et al., 2010), Bramhall et al. (2014) used a relatively lower gentamicin dose (0.05 mM) and observed a similar level of OHC toxicity. Although Bramhall et al. (2014) exposed the cochlear explants to gentamicin (0.05mM) for only 16 hours, they cultured the explants for an additional 72 hours in a drug-free media. Since they allowed a longer duration for the development of gentamicin-induced OHC loss in the cochlear explants following the gentamicin exposure, this might explain why they observed a greater gentamicin-induced OHC toxicity compared to our study and the *in-vitro* studies discussed above (Mazurek et al., 2012; Nakamagoe et al., 2010). Hence, the method of culturing the inner-ear explants in drug-free media after aminoglycoside exposure could be

applied in future studies that aim to investigate the long-term ototoxic effects of aminoglycosides.

Similar to how we observed a relatively lower level of gentamicin-induced cochlear toxicity, we also observed a lower level of gentamicin-induced vestibular toxicity when compared to another *in-vitro* study (Kim et al., 2009). Kim et al. (2009) observed approximately 83% utricular HC loss in the utricular explants of Sprague-Dawley rats (P 2-4) that were treated with gentamicin (1M) for 24 hours (n = 5). Meanwhile, we only observed about 68% utricular HC loss in the utricular explants treated with gentamicin (2.4 mM) for 24 hours. Hence, although the utricular explants were exposed to gentamicin for the same duration, Kim et al. (2009) observed a greater level of utricular HC loss despite using a lower gentamicin dose, when compared to our study.

Kim et al. (2009) mentioned that they exposed the explants to gentamicin on the first culture day. Since they did not specify the duration of organ culture before aminoglycoside exposure, it is likely that they did not incubate the utricular explants in a drug-free culture media before exposing the explants to gentamicin (1mM). Forge et al., (2000) reported that they cultured the utricular explants in a drug-free culture media before the aminoglycoside treatment to stabilize any surgical trauma induced during the isolation. For the same purpose, the inner ear explants were cultured for 24 hours in drug-free culture media before they were exposed to the aminoglycosides on the second culture day, in our experiment. Hence, by adding aminoglycosides to the culture on the first day and thereby limiting the chance for the utricular HCs to recover from the surgical trauma, the dissection and subsequent surgical trauma could have played a relatively larger role in lowering HC survival besides aminoglycoside exposure in Kim et al. (2009)'s study when compared to our

study. This might explain why Kim et al. (2009) observed greater gentamicin-induced vestibular damage compared to our study.

The *in-vitro* studies discussed above (Bramhall et al., 2014; Kim et al., 2009; Lee et al., 2013; Nakamagoe et al., 2010) have evaluated gentamicin-induced toxicity in either the cochlea or the vestibular system but not both. Since each study has different methods, it is difficult to compare the cochlear and vestibular toxicity between the *in-vitro* studies. For example, compared to Nakamagoe et al. (2010)'s study, which evaluated gentamicin-induced cochlear toxicity, Kim et al. (2009) used half the duration of aminoglycoside exposure (48 versus 24 hours, respectively) but a 10-fold greater gentamicin dose (0.1mM versus 1mM) for evaluating gentamicin-induced vestibular toxicity. *In-vitro* studies like Ding et al. (2002) that evaluate both gentamicin-induced cochlear and gentamicin-induced vestibular toxicity are severely lacking. However, despite evaluating the gentamicin-induced cochlear and vestibular toxicity simultaneously, Ding et al. (2002) did not directly compare the gentamicin-induced cochlear and vestibular toxicity. In this sense, our study is unique as it has evaluated and compared gentamicin-induced cochlear and vestibular toxicity.

Upon treating the cochlear explants of C57/10J mice (P2-P3) with gentamicin (1 mM) for 24 hours, Ding et al. (2002) observed approximately 50% IHC loss. Using linear regression, we estimated that gentamicin (0.8 mM) could result in 50% IHC loss. Hence, compared to Ding et al., (2002)'s study, we used a slightly lower gentamicin dose (1 versus 0.8mM) and achieved a similar level of IHC toxicity. However, Ding et al., (2002) observed approximately 95% OHC loss in the cochlear explants treated with gentamicin (1 mM) while we observed approximately 84% OHC loss in the cochlear explants treated with gentamicin 2.4 mM. Hence, Ding et al. (2002) observed a greater OHC loss even after using more than 2-fold lower gentamicin dose when compared to

our study (1 mM versus 2.4 mM, respectively). Also, they observed 70% utricular HC loss in the utricular explants treated with gentamicin (1 mM) while we observed only 68% in the utricular explants treated with gentamicin (2.4 mM). Hence, Ding et al. (2002) observed a similar level of utricular HC loss despite using less than half the gentamicin dose used in our study (1 mM versus 2.4 mM, respectively). In short, we observed a comparable gentamicin-induced IHC toxicity but substantially lower gentamicin-induced OHC and utricular HC toxicity compared to Ding et al. (2002)'s study.

There are many similarities when comparing the methods of our study with that of Ding et al. (2002)'s such as the age of the animals used (P2-P3 versus P2-P4), the use of collagen gel for organ culture, the duration of organ culture before aminoglycoside exposure (24 hours) and the duration of aminoglycoside exposure (24 hours). However, there is a difference between the species of animal used as Ding et al. (2002) used C57/10J mice while we used Wistar rats. Even so, it is surprising that we observed relatively lower aminoglycoside-induced ototoxicity in the rats used in our study when compared that in the C57/10J mice used in Ding et al. (2002)'s study as previous literature suggests that rats are more susceptible to aminoglycoside-induced ototoxicity than C57/10J mice (Wu et al., 2001).

Interestingly, Wubbels (2003) pointed out that the HC density in the control utricular explants of Ding et al. (2002)'s study was exceptionally low when compared to that of other studies evaluating HC density in the utricle. Furthermore, we counted approximately 1830 utricular HC per mm$^2$ in our control utricular explants (122 utricular HCs per 0.07 mm$^2$) while Ding et al. (2002) counted 344 utricular HCs per mm$^2$ (74.9 utricular HCs per 0.22 mm$^2$) in their control utricular explants. Hence, it is possible that besides the aminoglycoside exposure, surgical trauma or culture contamination could have played a role in reducing the HC survival in Ding et al. (2002)'s study

and this might be one of the reasons why they observed a greater level of gentamicin-induced HC damage compared to our study. Nonetheless, since *in-vitro* studies evaluating and comparing gentamicin-induced cochlear and vestibular toxicity are extremely limited, more such studies are needed to delineate gentamicin's ototoxicity *in-vitro*.

### 4.2) Amikacin-induced ototoxicity

Compared to the *in-vitro* studies investigating gentamicin-induced cochlear toxicity, the *in-vitro* studies evaluating amikacin-induced cochlear toxicity are limited. Nonetheless, we observed a lower level of amikacin-induced IHC and OHC damage when compared to a study by Kim et al. (2016) which evaluated amikacin-induced cochlear toxicity *in-vitro* and this disparity might be due to the differences in the species of the animal used and the duration of amikacin exposure.

Kim et al. (2016) found that the amikacin (0.5 mM) treatment resulted in approximately 48% OHC loss in the cochlear explants of the institute for research (ICR) mice (n = 4). In contrast, we observed a maximum of only 20% amikacin-induced OHC loss following the treatment with the maximum amikacin dose (2.4 mM) used in our study. Hence, despite using a 5-fold lower amikacin dose (0.5 mM), Kim et al. (2016) was able to achieve more than twice the OHC loss compared to our study. Furthermore, Kim et al. (2016) also found that amikacin (1 mM) treatment could result in approximately 89% IHC loss in the cochlear explants of ICR mice (n = 4). Meanwhile, in our study, we observed approximately 63% IHC loss in the cochlear explants treated with amikacin 1.2 mM. Hence, compared to our study, Kim et al. (2016) achieved a greater amikacin-induced IHC toxicity despite using a smaller amikacin dose (1mM

versus 1.2 mM). The differences in IHC and OHC toxicity could be explained by the difference in the type of animal used and the difference in duration of aminoglycoside exposure.

Kim et al. (2016) used ICR mice while we used Wistar rats (P2 to P4). Previous studies suggest that pre-treatment with antioxidant glutathione (GSH) can protect the cochlear HCs from aminoglycoside-induced toxicity (Nishida and Takumida, 1996). Since GSH expression is relatively lower in the blood plasma of ICR mice when compared to rats (Igarashi et al., 1983), it might be possible that the cochlea of the ICR mice received a lower supply of GSH and had a lower level of GSH in the cochlea before the dissection and therefore before the aminoglycoside-exposure. This could then result in reduced GSH-mediated protective effects following aminoglycoside exposure in the ICR mice explants compared to our rat explants. This might be one of the reasons why Kim et al. (2016) observed a greater IHC and OHC toxicity compared to our study.

Kim et al. (2016) exposed the cochlear explants to amikacin for 48 hours while we exposed the cochlear explants to amikacin for only 24 hours. As discussed previously, aminoglycoside-induced toxicity can increase drastically with increasing duration of aminoglycoside exposure (Lee et al., 2013). Hence, a relatively shorter duration of aminoglycoside exposure in our study could be yet another reason why we observed a lower IHC and OHC loss compared to Kim et al. (2016)'s study.

The *in-vitro* studies evaluating amikacin-induced vestibular toxicity are extremely limited. Using both Google scholar and PubMed search engines, only one such *in-vitro* study evaluating amikacin-induced vestibular toxicity was found (Bartolami et al., 2011). Bartolami et al. (2011) evaluated the ability of aminoglycosides to block the utricular HC stretch-activated channel and subsequent aminoglycoside-induced increase in potassium ions. They found that

amikacin (1mM) treatment could significantly increase the level of potassium ions in the utricular HCs. However, they did not evaluate amikacin-vestibular toxicity by counting the utricular HCs and so, comparisons could not be made with *in-vitro* studies evaluating amikacin-induced vestibular damage. Nonetheless, the lack of *in-vitro* studies evaluating amikacin-induced vestibular toxicity makes our study unique as we have evaluated amikacin-induced vestibular toxicity in addition to amikacin-induced cochlear toxicity.

### 4.3) Gentamicin exhibited greater cochleotoxicity compared to amikacin

The $TC_{50}$ of gentamicin for IHCs was approximately half of that of amikacin (400µM versus 0.9 mM, respectively). Meanwhile, the $TC_{50}$ of gentamicin for OHCs was also less than half of that of amikacin (1.14 mM versus >2.4 mM, respectively). This meant that the doses of gentamicin required to damage 50% of both the IHCs and OHCs were lower than that of amikacin. Furthermore, the type of aminoglycoside used (gentamicin or amikacin) had a significant effect on the IHC survival ($p = 0.0001$). Additionally, a significantly lower IHC survival rate was observed in the: gentamicin 0.6 mM treatment group when compared to the amikacin 0.6 mM treatment group ($p = 0.031$), and the gentamicin 0.9 mM treatment group when compared to the amikacin 0.9 mM treatment group ($p = 0.026$). Likewise, a significantly lower OHC survival rate was observed in the gentamicin 2.4 mM when compared to the amikacin 2.4 mM treatment groups ($p = 0.024$). These results showed that despite using equivalent doses, gentamicin treatment could result in a significantly lower IHC and OHC survival compared to amikacin treatment. Hence, the results indicate that gentamicin is more toxic to the cochlea than amikacin. This finding is in agreement with previous literature which demonstrated that gentamicin is more cochleotoxic than amikacin (Brummett et al., 1978; Kalkandelen et al., 2002; Kotecha and Richardson, 1994).

An early in-vitro study by Kotecha and Richardson (1994) ranked the cochlear toxicity of the various aminoglycosides and other molecules. They treated the cochlear explants of mice (day 1-2) with neomycin, dihydrostreptomycin, gentamicin, amikacin, spectinomycin, aminoglycoside, neamine and polyamine, spermine at concentrations of 0.25, 0.5 and 1.0 mM. The cultures were treated for 1 hour after which SEM and TEM were used to rank the morphological damage in the cochlea's apical and basal coils by order with "-" indicating no damage and "+", "+ +", "+++" and "++++" indicating increasing severity of cochlear damage with "++++" indicating complete damage.

Kotecha and Richardson (1994) categorized the gentamicin (0.25, 0.5 & 1 mM) induced OHC damage by "+", "++" and "+++", respectively in both the basal and apical coil. Meanwhile, they categorized amikacin (0.5 mM) induced OHC damage as "+" and amikacin (1 mM) induced OHC damage as "++" in the basal coil. Amikacin (0.25 mM and 0.5 mM) treatment did not show any signs of damage in the apical coil while mild (+) damage was observed from amikacin (1M) treatment in the basal coil. Overall, their results indicate that gentamicin is more toxic to the cochlea than amikacin, however, the use of an ordinal scale makes it difficult to directly compare the toxic effects of the different aminoglycoside doses with our study.

An in-vivo study by Brummett et al. (1978) evaluated the cochlear toxicity of various aminoglycosides: tobramycin, gentamicin, amikacin, and sisomicin. For this purpose, the guinea pigs were subjected to daily subcutaneous administration of the aminoglycosides (0, 50, 100, 150, or 200mg/kg) for a duration of 4 weeks. They found that both gentamicin 50 mg/kg and amikacin 100 mg/kg treatment resulted in nearly 50% OHC loss. From this result, we can observe that, when compared to gentamicin, twice the dosage of amikacin was required to achieve a similar level of

cochlear toxicity. Hence, the result from Brummett et al. (1978)'s study supports our finding that gentamicin is more toxic to the cochlea than amikacin.

Another in-vivo study by Kalkandelen et al. (2002) compared the cochlear toxic effects of four aminoglycosides, streptomycin, gentamicin, amikacin, and netilmicin. The aminoglycosides were administered to guinea pigs at respective doses of 37.5, 50, 125, or 150 mg/kg via the trans-tympanic route, twice a day for a duration of one week. They found that the gentamicin (50mg/kg) resulted in slightly more damage than the amikacin (150mg/kg), although the amikacin dose used was 3-fold greater than the dose of gentamicin. Therefore, the findings from Kalkandelen et al. (2002)'s study also suggest that gentamicin is more cochleotoxic than amikacin.

In all of the studies discussed in this section, the data were measured using an ordinal scale and thus, the aminoglycoside damage observed in the studies are subjective. This might introduce observational bias which might make the results from the studies less accurate.

**4.4) Gentamicin resulted in greater vestibulotoxicity compared to amikacin**

The $TC_{50}$ of gentamicin for utricular HCs was lower than that of amikacin (2.09 mM versus >2.4 mM, respectively). This result meant that the dose of gentamicin required to kill 50% of the utricular HCs was lower than that of amikacin. Furthermore, the type of aminoglycoside used (gentamicin or amikacin) had a significant effect on the utricular HC survival ($p < 0.0001$). Additionally, a significantly lower utricular HC survival rate was observed in the: gentamicin 0.6 mM treatment group when compared to the amikacin 0.6 mM treatment group ($p = 0.0265$), the gentamicin 0.9 mM treatment group when compared to the amikacin 0.9 mM treatment group ($p = 0.0208$), the gentamicin 1.2 mM treatment group when compared to the amikacin 1.2 mM treatment group ($p = 0.0029$), and the gentamicin 2.4 mM treatment group when compared to the

amikacin 2.4 mM treatment groups ($p < 0.0001$) (Figure 12A). These results demonstrated that despite using equivalent doses, gentamicin treatment could result in a significantly lower utricular HC survival compared to amikacin treatment. Hence, the results indicate that gentamicin is more toxic to the vestibular system than amikacin. This finding is in agreement with the observations made in Christensen et al. (1977), Yian and Xiaodong (1995), and Selimoğlu et al. (2003)'s study, all of which concluded that gentamicin was more vestibulotoxic than amikacin.

Christensen et al. (1977) compared the ototoxic potential of gentamicin and amikacin in mongrel cats. Animals received subcutaneous injections of amikacin (90 mg/kg or 45 mg/kg) or gentamicin (18 mg/kg or 9 mg/kg) once a day for a week. The vestibular functions, maintenance of normal gait and normal righting effect, were evaluated following the treatment. Evidence for ataxia was evaluated 5 to 6 hours after each dosing. They reported that none of the cats showed behavioral signs of vestibular dysfunction in the amikacin 45 mg/kg and 90 mg/kg groups. In contrast, all animals in the gentamicin 9 mg/kg and 18 mg/kg groups were found to exhibit signs of vestibular function loss. Hence, the findings from Christensen et al. (1977)'s study indicate that gentamicin is more vestibulotoxic than amikacin and this finding was further supported by Yian and Xiaodong (1995)'s study which also concluded that gentamicin was more toxic to the vestibular system than amikacin.

A study by Selimoğlu et al. (2003) compared the vestibulotoxic effects of the most commonly used aminoglycosides such as streptomycin, gentamicin, amikacin, and netilmicin in pigmented guinea pigs. They administered streptomycin (125 mg/kg), gentamicin (50 mg/kg), amikacin (150 mg/kg), and netilmicin (37.5 mg/kg) via the peritoneal route to one group of guinea pigs and administered the aminoglycosides at 0.25 ml/kg in a 4% saline solution (40 mg/ml) via the transtympanic route to another group of guinea pigs, for the duration of 7 days. The vestibular

organs were then removed and hematoxylin and eosin staining was performed after which the explants underwent optical microscopic examinations. The absence of hydropic and vacuolar degeneration and loss of hair cells were noted and ranked via an ordinal scale as "mild change", "moderate change", "severe change" and "very severe changes". They found that streptomycin caused the most damage to the vestibular system followed by gentamicin, amikacin, and netilmicin. They did not find any statistically significant difference in the severity of the vestibular damage between the two different administration routes (peritoneal versus transtympanic). Interestingly, they mentioned that amikacin (150 mg/kg) caused mild to moderate vestibular damage while gentamicin (50 mg/kg) caused moderate to severe vestibular damage (n = 10) so, from their result, it appears that gentamicin is more toxic to the vestibular system compared to amikacin even when the concentration of gentamicin is 3-fold lower than amikacin. Hence, the results from Selimoğlu et al. (2003)'s study also support our finding that gentamicin is more vestibulotoxic than amikacin.

**4.5) Amikacin treatment caused cochlear but not vestibular toxicity.**

Amikacin treatment did not reduce the survival rate of utricular HC by more than 12% of control at any of the doses used. Furthermore, the amikacin doses did not have a significant effect on the survival of the utricular HCs ($p = 0.549$). Hence, these results suggest that amikacin is not toxic to the vestibular system. Similarly, amikacin treatment did not reduce the survival rate of the OHCs by more than 20%, at any of the doses tested and the amikacin doses did not have a significant effect on the OHC survival ($p = 0.645$). Thus, these results imply that amikacin was not toxic to the OHCs. Meanwhile, we observed a decreasing trend in the IHC survival with increasing amikacin doses and the amikacin doses were found to have a significant effect on the

IHC survival (p = 0.0245). Furthermore, we found that the amikacin 0.9 mM treatment could result in considerable IHC damage, that is, a 50% reduction in IHC survival and a significant decrease in IHC survival was observed in the amikacin 2.4 mM treatment group when compared to the amikacin 0.3 mM treatment group (p = 0.0067), the amikacin 1.2 mM treatment group when compared to the amikacin 0.6 mM treatment group (p = 0.0261) and the amikacin 2.4 mM treatment groups when compared to the amikacin 0.6 mM treatment group (p = 0.0172). Hence, these results imply that amikacin is toxic to the IHCs and since IHCs are crucial for the physiological role of the cochlea (that is, sound detection and transduction), the results indicate that amikacin can impair cochlear functions. Hence, the results discussed above indicate that amikacin is toxic to the cochlea but not to the vestibular system. This finding is in agreement with the *in-vivo* studies (Freeman et al., 2001; Kitasato et al., 1990) which demonstrated that amikacin was toxic to the cochlea but not the vestibular system.

An *in-vivo* study by Kitasato et al. (1990) compared the ototoxicity of nine different aminoglycosides: ribostamycin, dactinomycin, dibekacin, kanamycin, amikacin, netilmicin, tobramycin, gentamicin, and sisomicin. The ototoxicity of gentamicin and amikacin will be discussed in this section. The aminoglycosides gentamicin (50, 75, and 100 mg/kg day) and amikacin (100 and 200 mg/day) were intramuscularly administered to guinea pigs for the duration of 4 weeks. The pinna reflex response is the twitching response of the external ear that is produced by rodents upon hearing a noise and loss of the pinna reflex response when exposed to sound stimuli indicates hearing loss. Thus, the pinna reflex response was evaluated twice a week with the help of an audiometer to evaluate cochlear toxicity. After the last treatment, the animals were sacrificed and the cochlea and vestibular organs (superior, posterior, and anterior crista ampullaris, utricle, and saccule) were isolated. The cochlear and vestibular explants were stained with

hematoxylin and eosin and subjected to histological examination under a light microscope during which histological abnormalities such as the loss of OHCs in the cochlea and the loss of HCs and smooth epithelial surface, and vacuolar formation in the vestibular system organs were noted and ranked as slight, moderate or severe.

Kitasato et al. (1990) found that three out of five animals in the amikacin (100 mg/kg/day) treatment group and four out of six animals in the gentamicin (100 mg/kg/day) treatment group did not produce a pinna reflex response when presented with the maximum sound frequency of 20kHz. These results demonstrate severe hearing loss induced by amikacin and gentamicin. Moreover, they found that 15 out of 36 (41.6%) cochlear samples from animals treated with amikacin 100 mg/kg/day exhibited histological abnormalities. Likewise, 9 out of 16 (56.3%) cochlear samples from animals treated with amikacin 200 mg/kg/day also exhibited histological abnormalities. In contrast, none of the utricular (0/9) and saccular (0/8) samples from amikacin (100 mg/kg/day) treated animals, and similarly, none of the utricular (0/4) and saccular (0/4) samples from the amikacin (200 mg/kg/day) animals, showed any evidence of histological damage. Likewise, histological damage was also absent in all crista ampullaris samples (0/28) from animals receiving amikacin 100 (mg/kg/day) while a single posterior crista ampullaris among a total of 12 crista ampullaris samples (4 anterior and posterior crista ampullaris, and 3 posterior crista ampullaris) from animals receiving amikacin 200 (mg/kg/day) showed a slight histological abnormality. Thus, the results from the Kitasato et al. (1990)'s study support our finding that amikacin treatment is toxic to the cochlea but not to the vestibular system.

A study by Freeman et al. (2001) aimed to investigate and compare the cochlear and vestibular toxicity of amikacin. For this purpose, guinea pigs were administered with amikacin (450mg/kg) via intramuscular injections for 5 days per week until the disappearance of the Preyer

reflex (average of 8 +/- 2 days). Following the amikacin treatment, the auditory function was evaluated using short-latency auditory nerve brainstem evoked responses (ABR) and the vestibular functions were evaluated by measuring the short-latency vestibular evoked potentials (VsEP) separately from the utricle, saccule, and lateral semicircular canal. The VsEPs were measured by presenting impulses of linear and horizontal acceleration to the rigidly held head of the animal, in the linear and horizontal plane stimulation of the utricle and the saccule, respectively. VsEP outputs from the utricle (x-VsEP) and the saccule (y-VsEP) were recorded by using a subdermal needle electrode (Grass) positioned at the different earlobes. Similarly, angular acceleration was presented in a clockwise direction to the animals and the VsEP produced from the lateral semicircular canal (a-VsEP) was recorded using an Amplaid EMG-14 evoked potential system.

Freeman et al., (2001) found that the ABR threshold, that is, the minimum sound intensity required to produce an ABR signal, in the control animals was 59.3 ± 5.1 dB peak equivalent (pe) sound pressure level (SPL). However, they found that the ABR threshold was increased to approximately 100 dB pe SPL in two amikacin (450 mg/kg) treated animals, while the rest of the ten amikacin (450mg/kg) treated animals did not produce an ABR even after being presented with the maximal sound intensity (135 dB pe SPL) (n = 10). This indicated a complete loss of cochlear function in approximately 82% of the amikacin (450 mg/kg) treated animals. Interestingly, the x-VsEP, y-VsEP, and a-VsEPs values were not significantly different before and after amikacin treatment (n = 12). These results indicated that the functions of vestibular organs, the utricle, the saccule, and the lateral semi-circular canals, were not damaged following amikacin treatment. Therefore, this study suggests that amikacin is toxic to the cochlea but not the vestibular system.

**4.6) Gentamicin appeared to be more toxic to the cochlea than the vestibular system.**

Gentamicin doses were found to have a significant effect on the IHC, the OHC, and the utricular HC survival (p = 0.0013, 0.0267, and p = 0.0120, respectively). However, we found that the $TC_{50}$ of gentamicin for IHC was approximately 5-fold lower than the TC50 of gentamicin for utricular HC loss (0.4 mM versus 2.01mM, respectively). Likewise, the $TC_{50}$ of gentamicin for OHC was approximately 2-fold lower than that for utricular HC loss (1.14 mM versus 2.01mM, respectively). Thus, our results indicate that gentamicin is more toxic to the cochlea than to the vestibular system. This finding contradicts the observations made by previous studies which suggest that gentamicin is preferentially vestibulotoxic (Kitasato et al., 1990; Marais and Rutka, 1998). Hence, further studies are required to evaluate and compare the gentamicin-induced cochlear and vestibular toxicity *in-vitro*.

The comparison between the TC50s allowed us to compare the toxicity of different aminoglycosides (gentamicin versus amikacin) on separate inner ear organs (cochlea versus the vestibular system). By repeating the experiment at least three or more times, future studies could obtain three or more $TC_{50}$ values which could then be used to calculate the average and variance of the $TC_{50}$ values, for example, the average $TC_{50}$ for gentamicin-induced utricular HC toxicity +/- SEM, and analyze if an average $TC_{50}$ value is significantly different from another average $TC_{50}$ value, for example, the average $TC_{50}$ for gentamicin-induced OHC/IHC toxicity +/- SEM versus the average $TC_{50}$ for gentamicin-induced utricular HC toxicity +/- SEM. Following the example mentioned above, one could validate the observations made based on $TC_{50}$ values in our study such as whether or not gentamicin is more toxic to the cochlea than the vestibular system *in-vitro*.

**4.7) TGP treatment attenuated gentamicin-included utricular HC loss, but not cochlear HC loss**

A study by Jia et al. (2014) showed that 0.5-300 µg/ml TGP could exhibit significant anti-inflammatory properties in rat models of inflammation. Furthermore, we observed that TGP 100 µg/ml could significantly reduce gentamicin-induced HC death in our pilot experiment. Hence, the TGP (100 µg/ml) dose was chosen for the evaluation of TGP's protective effects against aminoglycoside-induced ototoxicity. Due to time constraints, we have evaluated the protective effects of TGP by using only a single dose of TGP (100µg/ml). Future studies could evaluate the protective effects of different doses of TGP. This would allow for a more detailed investigation of TGP's otoprotective effect as the correlation between the TGP doses and HC survival could be evaluated.

The gentamicin 0.3 mM + TGP 100µg/ml dose was chosen because approximately 50% IHC damage was observed in the gentamicin 0.3 mM treatment group, so we wanted to compare the effects of gentamicin 0.3 mM + TGP100µg/ml with the effects of gentamicin 0.3 mM treatment alone, on IHC survival to investigate if TGP could prevent gentamicin induced IHC damage.

Similarly, gentamicin 0.9 mM + TGP 100µg/ml was chosen because approximately 50% OHC damage was observed in the gentamicin 0.9 mM group, and we wanted to compare the effects of gentamicin 0.9 mM + TGP100µg/ml with the effects of gentamicin 0.9 mM treatment alone on OHC survival to evaluate if TGP could prevent gentamicin induced OHC damage.

Likewise, amikacin 0.6 mM + TGP 100µg/ml was chosen because approximately 50% IHC damage was observed in the amikacin 0.6 mM treatment group and we wanted to compare the effects of amikacin 0.6 mM + TGP100µg/ml with the effects of amikacin 0.6 mM treatment alone on HC survival, to evaluate if TGP could prevent amikacin induced IHC damage.

As for the dose for combination treatment in utricular explants, gentamicin 2.4 mM + TGP100μg/ml was chosen because nearly 50% utricular HC death was observed in the gentamicin 2.4 mM treatment group, so we wanted to compare the effects of gentamicin 2.4 mM + TGP100μg/ml with the effects of gentamicin 2.4 mM treatment alone on utricular HC survival to investigate if TGP could prevent gentamicin-induced utricular HC damage.

We did not observe considerable OHC damage or utricular damage for amikacin since the survival rate for OHC and utricular HC at the maximum amikacin dose (amikacin 2.4 mM) was nearly 79% and 88%, respectively, so, we did not choose amikacin + TGP doses to investigate if TGP could reduce amikacin-induced OHC or utricular HC death as there was not much amikacin-induced OHC or utricular HC death in the first place.

The previous literature suggests that the basilar HCs might be more susceptible to surgical trauma compared to the HCs in the middle and apical regions of the cochlea, during the dissection (Sha et al., 2001; Zajic and Schacht, 1987). Upon re-analyzing our data, we found instances where minimal HC loss was observed in both the apical and mid region while near complete HC loss was observed in the base region. Although aminoglycoside-induced HC survival varies according to the regions of the cochlea (Sha et al., 2001), in instances where the HC survival does not decrease progressively from the apical to the basal region and large variations in HC loss (minimal versus complete HC loss) are observed between the base versus apical and middle regions, surgical trauma could have had a confounding effect on the survival of basilar HCs after aminoglycoside exposure. Hence, the exclusion criteria were optimized as follows: in case a complete HC loss was observed in the basal region in contrast to both the apical and middle region where only minimal HC loss was observed, then the HC count data

from the basal region of the cochlea were excluded and, the HC count was excluded from any region where surgical trauma was observed.

Following the optimization of the exclusion criteria, the average IHC survival appeared to increase in the amikacin 0.6 mM treatment group and the average OHC survival increased in the gentamicin 0.9 mM treatment group. Hence, for the dose of aminoglycoside to be used in combination with TGP, future studies could choose aminoglycoside doses resulting in comparable IHC/OHC loss. This would ensure that any difference in the HC loss observed between the different aminoglycoside + TGP treatment groups was not because the aminoglycoside doses used resulted in different levels of HC toxicity. For example, it might be possible that TGP could ameliorate medium HC loss but not severe HC loss or vice versa.

There was a significant increase in utricular HC survival rate (by approximately 57%) in the gentamicin 0.9 mM + TGP 100μM group when compared to that of the gentamicin 0.9 mM alone group. This result indicated that TGP treatment could attenuate gentamicin-induced utricular HC loss.

Previous studies have implicated inflammation as one of the mechanisms behind aminoglycoside-induced vestibular toxicity (Warchol, 1999). A study by Lin et al. (2017) suggested that TGP could reduce inflammation in a rat model of inflammatory bowel disease by inhibiting the level of pro-inflammatory cytokines IL-17, TNF-α, and IL-6 expression levels and, increasing the expression of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) in the colon cells and plasma of 6-8 weeks old Sprague-Dawley rats. Furthermore, they also suggested that TGP could reduce the percentage of the TH17 cells, the pro-inflammatory cells which when overexpressed can result in the development of inflammatory disease (Gaffen, 2008), while increasing the percentage of Treg cells, the anti-inflammatory cells that help to resolve

inflammation (Eastaff-Leung et al., 2010). The finding by Lin et al. (2017) is supported by a study from Cao et al. (2011), which suggested that the main active component of TGP, paeoniflorin, could protect mice against a lethal LPS challenge by inhibiting LPS-stimulated pro-inflammatory cytokines TNF- α and IL-1β production, while accelerating anti-inflammatory cytokine IL-10 expression. Hence, TGP could have significantly increased the utricular HC survival (% of control) by resolving the gentamicin-induced inflammation via the downregulation of pro-inflammatory cytokine expression and upregulation of anti-inflammatory cytokine expression.

Although previous studies have suggested inflammation as one of the mechanisms behind aminoglycoside-induced cochlear toxicity (Garcia-Alcantara et al., 2018; Sun et al., 2015) and vestibular toxicity (Warchol, 1999), we found that the protective effect of anti-inflammatory TGP was limited to the vestibular system. This finding suggests that the underlying mechanisms of gentamicin-induced toxicity may be different between the cochlear and vestibular systems.

**4.8) Limitations and Future Directions**

Due to the limited time and a large number of treatment groups in our study, the sample size in each group was limited to three. Type-2 error refers to the error of failing to reject the null hypothesis when the null hypothesis is false. This type of error can occur during the analysis of data with low sample sizes. Thus, type-2 error could have prevented us from finding significant differences between the aminoglycoside + TGP and aminoglycoside alone treatment groups even if they existed.

Although three or more areas were analyzed throughout the cochlea, there were instances where we were unable to analyze HC survival rate in a particular region of the cochlea (apex or middle, or base) which, due to the limited sample size in our study, meant that there were not enough sample areas from each region of the cochlea to analyze the HC survival rate in the

cochlear regions (apex, middle and base), separately. Therefore, for individual cochlear explants, we averaged the IHC and OHC survival rates from all regions to represent the overall IHC and OHC survival rate in the cochlea. Although we could not find a significant difference in the overall IHC/OHC survival rate throughout the cochlea, there may be a significant difference in IHC/OHC survival rate in one or more cochlear regions (apex, middle or base) of the aminoglycoside alone treatment groups when compared to aminoglycoside + TGP treatment groups. Therefore, the sample size could be increased for each treatment group to reduce the chance of type-2 error and to allow for a separate analysis of HC survival in the different regions of the cochlea, as there is evidence that the HCs in the different regions of the cochlea (apex, middle and base) exhibit different sensitivity to aminoglycoside-induced toxicity (Lee et al., 2013).

Currently, there are no studies evaluating and comparing aminoglycoside-induced cochlear inflammation with aminoglycoside-induced vestibular inflammation using the same method. For example, previous studies such as Sun et al. (2015) and Garcia-Alcantara et al. (2018) evaluated the aminoglycoside-induced cochlear inflammation in mice cochlea while the previous study such as Warchol (1999) evaluated aminoglycoside-induced vestibular inflammation in chick utricle. Thus, the difference in aminoglycoside-induced cochlear versus vestibular toxicity between the studies might simply be due to the difference in the animal species used since the avian species can regenerate HCs after aminoglycoside-induced damage while HC regeneration after aminoglycoside-induced damage is relatively limited and not as efficient in the mammals (Benkafadar et al., 2021). It might be possible that aminoglycoside-induced inflammation is more predominant in the vestibule than in the cochlea. This could explain why the anti-inflammatory TGP was more effective at ameliorating aminoglycoside-

induced vestibular toxicity than aminoglycoside-induced cochlear toxicity. Hence, future studies could evaluate and compare the TGP's anti-inflammatory effects on aminoglycoside-induced cochlear versus aminoglycoside-induced vestibular inflammation. This could be done by measuring the activation of inflammatory pathways and resident cochlear macrophages, and evaluating the expression of inflammatory cytokines.

Previous studies have shown that TGP can reduce inflammation by blocking an inflammatory pathway, that is, the Nf-kB pathway (Gu et al., 2017; Naveed et al., 2018) so, the effects of aminoglycosides on activation of the NF-kB pathway and subsequently, the effects of TGP on aminoglycoside-induced activation of the NF-kB pathway, can be evaluated by using immunochemistry as described by Naveed et al. (2018).

Aminoglycoside-induced activation of resident macrophages and changes in inflammatory cytokine expression were identified as some of the mechanisms behind aminoglycoside-induced toxicity in the cochlea (Garcia-Alcantara et al., 2018; Sun et al., 2015). Investigation of such inflammatory processes in the utricle following aminoglycoside exposure is limited. Thus, future studies could investigate if aminoglycosides and TGP can change the expression of pro-inflammatory and anti-inflammatory cytokines, and subsequently affect the activation/expression of resident macrophages in the cochlear and vestibular explants. The changes in inflammatory cytokine can be investigated by using the enzyme-linked immunosorbent assay (ELISA) procedure as previously described by Sun et al. (2015) and the activation of resident utricular macrophage can be explored by IBA-1 staining as previously described by Garcia-Alcantara et al. (2018).

# Conclusion

In conclusion, our results suggest that: amikacin was toxic to the cochlea but not to the vestibular system; gentamicin was more toxic to the cochlea relative to the vestibular system; gentamicin was more cochleotoxic and vestibulotoxic compared to amikacin; and TGP treatment could attenuate aminoglycoside-induced vestibular toxicity but not aminoglycoside-induced cochlear toxicity.

Although we generally observed a relatively lower aminoglycoside-induced ototoxicity compared to other *in-vitro* studies, most of our findings were in agreement with the *in-vivo* studies. This suggests that the *in-vitro* inner ear organ culture used in this study can be used as a useful tool to screen and compare cochlear and vestibular toxicity for new aminoglycosides.

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